STUDIES ON ENDOPHYTIC FUNGI HARBORED IN *NOTHAPODYTES FOETIDA* PLANTS

SUMMARY
THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

AGRICULTURAL MICROBIOLOGY

TO

DEPARTMENT OF AG. MICROBIOLOGY
FACULTY OF AGRICULTURAL SCIENCES
ALIGARH MUSLIM UNIVERSITY, ALIGARH (INDIA)

By

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The thesis embodies the work on the chemical investigation of fungal endophytes harboured in medicinal plant, *Nothapodytes foetida* (Icacinaceae), native to Western Ghats. It includes a short review of literature of various constituents isolated from different endophytes. It also includes the isolation of camptothecin, a naturally occurring alkaloid and an important precursor of anticancer drugs, from plant as well as from a new source "endophytic fungus." Furthermore, it also describes the isolation of two known and three new metabolites from this novel endophyte. Manuscript of the thesis comprises of five chapters.

The first chapter of this dissertation presents general introduction about fungal endophytes and an overview of different facets investigated in this study with emphasis on the natural precursor of anticancer drug camptothecin. The second chapter has been devoted to the summary statement of a review on few representative groups of fungal endophytes, their associated secondary metabolites and a chemical and biological profile of some important leads. In addition, a brief description of diversity of fungal endophytes has also been given.

The third chapter, which forms the major part of the thesis, deals with the isolation of endophytes from *Nothapodytes foetida* plants obtained from various locations and biological studies of a selected fungal endophyte-RJMEF001, later on identified as *Entrophospora infrequens*. Even when the present investigations have resulted in the isolation of a number of endophytic fungi from the *Nothapodytes foetida* plant, the selected fungus is reported here to produce camptothecin and its analogues when grown in semi-synthetic liquid medium. Besides, detailed shake flask studies under different culture conditions have been discussed. In addition to that optimization of media and identification of the organism, using different microscopic as well as molecular techniques have also been discussed in detail. Further, preliminary precursor and solid-state fermentation studies have also been described. This chapter includes the scale up studies for the biomass as well as camptothecin production in bioreactors.

The fourth chapter deals with the experimental details and the spectral data of the constituents isolated from the different solvent extracts of mycelia of *E. infrequens*. Further more, an attempt has been made to assess the *in vitro* cytotoxicity of fungal

extracts made by organic solvent extraction, chloroform: methanol and butanol as well as the fractions eluted in benzene, ethyl acetate, and methanol. The *in vitro* cytotoxicity of the fungal extracts evaluated using 10 human cancer cell lines (Hep-2: liver, SW-620, HCT-15, 502713, HT-29: colon, SNB-78: CNS, DU-145: prostate, KB: oral, A-549, H226: lung) at 10 and 30 μgml^{-1} doses is also described as a bioassay in support of the qualitative presence of CPT. An elaborate use of chromatography and spectroscopy has been made for the isolation and structure determination of the compounds. The present investigations have resulted in isolation of six molecules such as camptothecin (CPT), 5-(hydroxymethyl)-2-furfuraldehyde, and ergosterol along with three other compounds, the structure of which indicates the novelty of these compounds to this fungus. The discussion on the entire set of results and the thesis emerging out of such results constitute the last chapter. At the end of the thesis, references to the relevant literature have been incorporated. Following is the summary of the work accomplished.

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial arena. Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism, product and utilitarian discovery in this field holds exciting promise. This is witnessed by the discovery of a wide range of microorganisms and the products thereof indicating these organisms as a promising resource for prospecting new chemical entities as future drugs.

The use of endophytic fungi for the production of secondary metabolites has been a priority regarded as an area of considerable interest, owing their enormous potential for production of bioactive compounds. The present study is based on the premise that screening of beneficial endophytic fungi possessing enhanced anticancer capabilities in conjunction with some additional traits viz. immunomodulators, antiviral, antibacterial, antifungal and radio protective compounds production may help provide certain strains with broad spectrum activities. The extended significance of the productivity of endophytes for these important bioactive metabolites lies in that, they provide an alternate strategy for easing the negative

impact of over exploitation of resource plant which are otherwise essential part of biodiversity and the ecosystem. As a poorly investigated store of microorganisms, hidden within the host plants, these endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with substantial medicinal and agricultural potential. Thus, the isolation, characterization and identification of such indigenously effective fungal flora with broad-spectrum biological activities are perceived as a viable and cost effective approach for developing a genetic resource.

In this study, the indigenous fungi have been isolated from wild *N. foetida* plant samples collected from Mahablashwer forest and grown successfully at RRL botanical garden of Jammu province (32^0 44 N' and 74^0 55' E, approximately 400m, altitude above see level) of J&K state, India in the year 1995-96.This important medicinal plant was for the first time explored for harbouring endophytes. Using skilled isolation procedures, 52 different endophytic fungal colonies were isolated from inner bark tissues of *N. foetida* explants. These fungal colonies were purified by routine single spore isolation, sub culturing and hyphal tip methods. All the isolates were screened for the production of anticancer alkaloid camptothecin through chemical profiling as well as by exhibiting innate potential of biological activities. One of the isolates (RJMEF001) from the inner bark tissues of *N. foetida* plant growing at RRL, Jammu was found to produce detectable quantities of CPT and its derivatives when grown in semi synthetic liquid medium. The fungal strain was characterized on the basis of its morphological and 16S ribosomal genetic markers. The scanning electron microscopy was used to record the characteristic features of the fungal spores. It demonstrated the prominent striations on the smooth surfaces of fungal spores, which are round to oval in shape. The morphological and phylogenetic analysis based on LSU rDNA typing, comparing the sequences in NCBI and ribosomal data banks suggested the identification of the strain RJMEF001 as *E. infrequens.* Sequence alignment with database showed that the strain is more close to *E. infrequens* (>98%) than the other close taxa e.g., *Rhizopus oryzae* strains UW FP973 and 846 (>97%). On the basis of morphological features, growth behaviour as well as DNA alignment homology, the organism is assumed to be *E. infrequens.* This promising isolate, *E. infrequens*-RJMEF001, was cultivated in various mycological broths viz; Czapek, Malt extract, Molasses, Goos

and Tschessch, Potato Dextrose, Ashner, and Kohn, Leonine, Bianchi, and Sabouraud broth supplemented with different salts and trace elements and designated as M_1, M_3, M_6, M_7, M_9, M_{10}, M_{11}, M_{12} and M_{18} respectively throughout in the present study, in order to optimize the medium for maximum growth and production of camptothecin. Significant quantitative and morphological changes were detected when the fungus was challenged to grow in different liquid media. The strain RJMEF001 exhibited substantial growth i.e. 28.48 ± 1.08 gl^{-1} in Czapek medium supplemented with Dextrose 30 gl^{-1} as a sole carbon and energy source and ammonium oxalate 3.0 gl^{-1} as nitrogen-source. But there was no detection of camptothecin in this broth. The camptothecin (489.42 ± 19.87 µg/100 g dry cell mass) was detected in culture filtrates of the fungus when grown in Sabouraud broth supplemented with trace elements magnesium sulphate (0.5gl^{-1}) and potassium dihydrogen orthophosphate (1.0 gl^{-1}). The CPT was also detected in varied amounts in cultures grown in liquid media viz. Goos and Tschessch (326.42 ± 36.55 µg), Leonine (200.34 ± 19.95 µg), and molasses (230.16 ± 25.88 µg) per 100 g dry cell mass. A range of carbon and nitrogen sources was screened for their capacity to support growth of *Entrophospora infrequens* and CPT production. There was no CPT formation in medium containing malt extract (M_3, M_{15}, M_{16}), Czapek (M_1, M_2, M_{13}, M_{14}), Ashner and Kohn and Bianchi liquid broths also did not support CPT production. The lowest cell mass (1.84 gl^{-1}) was obtained in high osmotic stress broth (M_{15}) using 10% NaCl. However, only traces of CPT were detected in potato dextrose broth. The highest CPT production of 503.07 ± 25.88 µg/100 g dry cell mass was obtained in Sabouraud broth with 1% (w/v) peptone and 4 %(w/v) dextrose. Sabouraud broth was found to be the best medium, among the various growth media tried for the production of camptothecin. The results on the growth kinetics in Sabouraud liquid medium indicated maximum dry biomass of 28.11 ± 1.92 gl^{-1} after 7 days when incubated at 28 ± 2°C. The results on the production kinetics indicated the maximum production of 0.575 ± 0.73 mg/100 g dry cell mass in 96 h incubation period. The generation time for the strain RJMEF001 was determined to be 14-16 h in sabouraud medium. Besides media optimization, the culture was also fed with known precursors of camptothecin. Analysis of the precursors data revealed the varied rates of CPT production in

Sabouraud broth supplemented with different precursor molecules. Interestingly, the endophytic fungal strain showed enhancement in the CPT yield in Sabouraud broth supplemented with tryptophan (10 mM) and tryptophan in combination with leucine (25 mM). The CPT yields were almost increased by 2 fold (estimated as 871.58 ± 86.2 µg/100 g dry cell mass and of 993.68 ± 99.3 µg/100 g dry cell mass at 96 h respectively) when tryptophan and tryptophan in combination with leucine were added to the medium. Furthermore, surface culture and solid-state fermentation studies were also conducted in order to optimize the culture method for optimum CPT production. The highest CPT production of 3.37 ± 0.44 mg/100 g dry cell mass with cell mass of 22.79 ± 0.8 gl^{-1} was obtained with surface culture fermentation in shake flaks at 21 days of incubation time. The results obtained during solid-state fermentation on moist wheat bran and wheat bran supplemented with sabouraud broth indicated maximum productivity of 200.33 ± 19.90 µg/100 g dry weight and 390.78 ± 20.10 µg/100 g dry weight respectively at 21 days. The results of this study therefore suggest that surface culture may be advantageous to submerged fermentation for cultivation of *E. infrequens* and production of CPT. In addition to the optimization of the fermentation parameters in shake flasks, an attempt has been made to scale up the process in a bench scale bioreactor for the production of camptothecin under submerged conditions. The maximum yield of CPT represents a productivity of 0.575 ± 0.031 mg/100 g dry cell mass at 96 h in shake flasks whereas 4.96 ± 0.73 mg /100 g dry cell mass was recorded in 48 h in a bioreactor. CPT was identified by various physico-chemical tests coupled with spectroscopic analyses and further confirmed by its biological assay.

The literature scan indicate that there is no published report about any endophytic microorganism or independently growing microbes producing CPT. We have for the first time now reported the results of present study (Puri *et al.* 2005). In this study the possibility of CPT being produced by one or more microbes associated with *N. foetidea* plants was also explored. The most logical place to focus the search for such microorganisms is one or more locations, which have naturally been supporting the growth of *N. foetida* for centuries. Thus, the indigenous isolates with the potential to produce novel bioactive molecules under a natural selection process

could be utilized for developing alternate natural sources for these important compounds like CPT and its analogues.

The present work also describes the isolation of camptothecin and 9-methoxycamptothecin as marker compounds for LC-MS studies by semi-preparative HPLC. The purity of the isolates was determined by LC-MS and other spectral analysis. Earlier reported procedures for isolation of 9-methoxy-camptothecin from the crude extract involved cumbersome procedures encompassing high-speed counter current chromatography and repeated crystallizations. In the present methodology, 9-methoxy-camptothecin has been isolated to 95% purity by semi-preparative HPLC from the mother liquor after crystallization of camptothecin from the crude extract of *N. foetida.*

CPT is a promising antitumour alkaloid. From a practical viewpoint; microbial fermentation as a means of producing bioactive substance has several advantages like industrial production of a bioactive substance like CPT requires reproducible, dependable productivity. If a microbe is the source organism, it can be grown in tank fermenters as needed, generating a virtually inexhaustible supply of CPT. Enhancing productivity being relatively easy in microorganisms, it can be a reliable, convenient and economical source for inexhaustible supply of CPT and its derivatives.

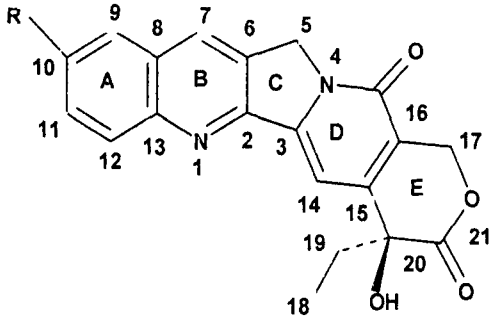
The extension of the study for exploration of other secondary metabolites with this endophyte has lead me to other minor metabolites which may be useful to trace the secondary metabolic pathway of this organism. Besides two known secondary metabolites ergosterol and 5-(hydroxymethyl)-2-furfuraldehyde, interestingly the present studies have made possible to isolate three new complex molecules showing marked anticancer activity. These compounds have been characterized as substituted phenoxy caffeic acid and corresponding peroxy ferulic acid ester of polysubstituted salicylic acid (Fig a-f).

Admittedly, the present study is only indicative of alternate source of Camptothecin supply for the production of potent anticancer analogue. Extensive study would be required to further develop the isolated fungal strain as a potential organism for the development of economical fermentation process either in surface culture or in

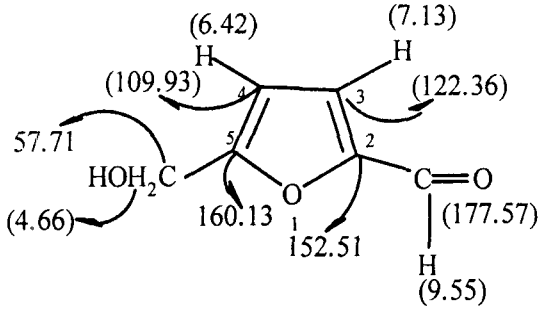
submerged deep tanks. Further more genomic and metablomic studies of the organism and the plant harbouring the organism would be interesting to know the secondary metabolite pathway used by the plant and microbes for generation of these important drug precursors.

Based on the HPLC, LC/MS, MS^2, ^{1}HNMR and ^{13}CNMR studies; structures of the six compounds isolated from the fungus have been characterized as shown (Fig. a-f) below.

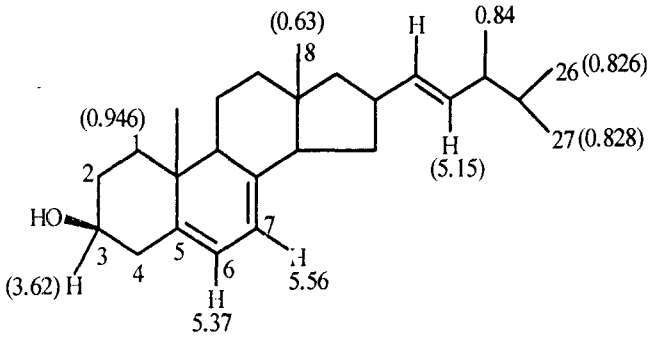
Known molecules



(a) Camptothecin, R=H

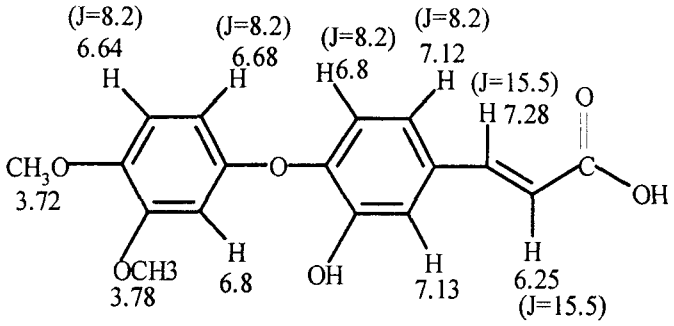


(b) 5-(hydroxymethyl)-2-furfuraldehyde

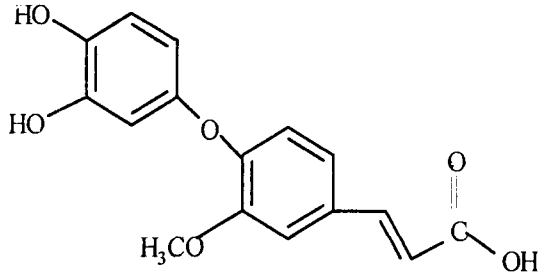


(c) Ergosterol

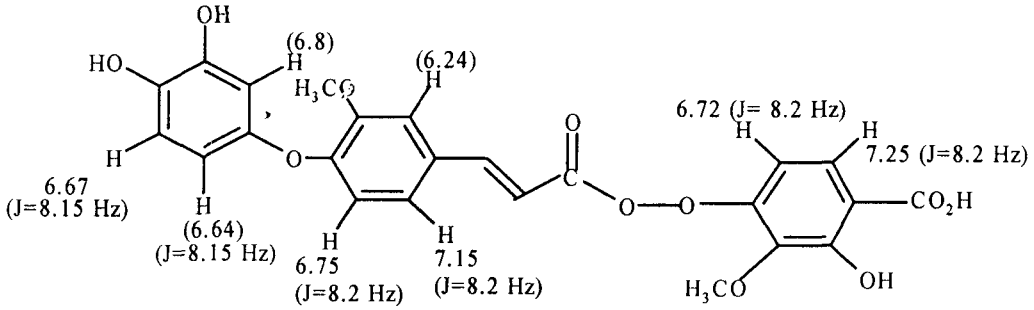
New molecules



(d) 4-(3, 4-dimethoxy-phenyl-1-oxy)-caffeic acid



(e) 4-(3,4-dihydroxy phenyl-1-oxy)-ferulic acid



(f) 4[4-(3,4-dihydroxyphenyl-oxy)-peroxy ferulyl]-3-methoxy-salicylic acid

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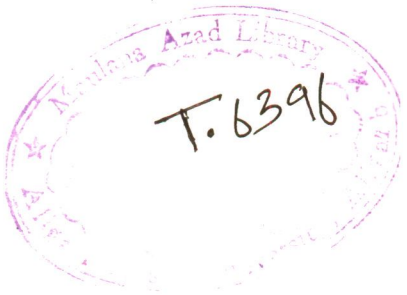

DEDICATED

TO

MY PARENTS

CERTIFICATE

This is to certify that the research work embodied in this thesis entitled "**Studies on endophytic fungi harbored in *Nothapodytes foetida* plants**" submitted to Aligarh Muslim University, Aligarh for the degree of Doctor of Philosophy in Agricultural Microbiology has been carried out by Miss Touseef Amna under our joint supervision. This is a bonafide work and has not been submitted in part or full to this or any other University/Institute. The thesis is suitable to be considered for the award of the degree of Doctor of Philosophy.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Studies on Endophytic fungi harbored in *Nothapodytes foetida* plants**" has been carried out by me at the Department of Biotechnology, Regional Research Laboratory (CSIR), Jammu.

Touseef Amna

(Touseef Amna)

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Praise to Almighty who bestows success and guides our destiny. I fail to find words to express my thankfulness and gratitude for his blessings and for bestowing me with perseverance in accomplishing this uphill task.

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of couple of years work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

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Whatever good I have in my life is a result of immense sacrifices made by my parents. I have no words to express my heartfelt gratitude and respect towards my parents for their blessings, endless love and constant inspiration throughout my studies. I offer hearty thanks to my beloved sisters Musarat and Insha whose unconditional love and affection has always been my strength and help me to buildup faith in myself throughout. My thanks are also due to my brother sayam for his endless love and affection.

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Again, a million of thanks to God, the Almighty who made me do this task and made every job a success for me.

Touseef Amna

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CONTENTS

ABBREVIATIONS

α	Alpha
β	Beta
δ	Delta-chemical shift downfield from TMS in ppm
Δ	Delta-the position of double bond
ε	Extinction coefficient
γ	Gamma
υ	Mu-frequency in cm^{-1}
λ	Wavelength in nm
μ	Micro
5-HMF	5-(hydroxymethyl)-2furfuraldehyde
b.p	Boiling point
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BtOH	Butanol
$CHCl_3$	Chloroform
CPT	Camptothecin
d	Doublet
dd	Double doublet
DDW	Double distilled water
DEPT	Distortionless enhancement by polarization transfer
DMH	Dextrose monohydrate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
E. infrequens	*Entrophospora infrequens*
EDTA	Ethylene diamine tetra-acetate
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide

EtOH	Ethyl alcohol
FAB-MS	Fast atom bombardment mass spectrometry
g	Gram
g^{-1}	Per gram
GC-MS	Gas chromatography mass spectrometry
h	Hours
HPLC	High performance liquid chromatography
Hz	Hertz
IR	Infra red
J_{Hz}	Coupling constant in Hertz
l	Litre
l^{-1}	Per litre
LC-MS	Liquid chromatography-mass spectrometry
m	Milli, Multiplet
M	Molar
m.p	Melting point
M^{+}	Molecular ion peak
MeOH	Methanol
mg^{-1}	Per milligram
min	Minutes
min^{-1}	Per minute
ml^{-1}	Per millilitre
MTCC	Microbial Type Culture Collection, IMTECH,Chandigarh
N. foetida	*Nothapodytes foetida*
ng	Nanograms
NMR	Nuclear Magnetic Resonance
^{o}C	Degree Celsius
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar

ppm/ppt	Parts per million/ Parts per trillion
q	Quartlet
rDNA	Ribosomal DNA
RJMEF001	Culture code
RNase	Ribonuclease
Rp	Reversed phase
rpm	Revolutions per minute
s	Singlet
SDS	Sodium dodecyl sulphate
Sol.	Solution
SRB	Sulforhodamine B dye
t	Triplet
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
v/v	Volume/volume
vol.	Volume
vvm	Volumes of air per minute per volume of broth
w/v	Weight/volume
Wt.	Weight

CHAPTER 1

GENERAL INTRODUCTION

It is estimated that approximately one quarter of the prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. The most popular analgesic, aspirin, was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources (Katzung, 1995; Pezzuto, 1996; Roberts, 1988). Morphine, the first alkaloid isolated more than 150 years back has stood the test of time and is still the drug of the choice. Today the largest users of traditional medicine are the Chinnese, with over 5000 plants and plant based products in their pharmacopeia (Bensky and Gamble, 1993). Natural-product based compounds have immense impact on modern medicine. For instance, about 40% of the prescription drugs are based on them. Besides, more than 50 % of the new chemical products registered with the FDA during 1981-2002 as anticancer agents, anti-migraine agents and antihypertensive agents were either natural products or derived thereof (Newman *et al.,* 2003). Plants also commonly act as hosts to a multitude of microbes including parasites, symbionts, endophytes, epiphytes and mycorrhizal fungi (Fisher and Petrini, 1990). These microorganisms may also influence the production of secondary plant metabolites, such as phytoalexins, whose presence can be triggered by elicitors from microbes (Bills, 1996). Such microbes may also be capable of producing secondary molecules identical with those produced by the plants.

Several hundreds of compounds with antibiotic activity have been isolated from microorganisms over the years, but only a few of them are clinically useful. The phenomenal success of penicillin led to the search for other antibiotic-producing microorganisms, especially from soil environments. One of the early successes (1943) was the discovery of streptomycin from a soil actinomycete, *Streptomyces griseus*. Actinomycetes, especially *Streptomyces* species, have yielded most of the antibiotics used in clinical medicine today. Some examples of antibiotics produced by the microorganisms are: Penicillin (*Penicillium chrysogenum*), Cephalosporin (*Cephalosporium acremonium*), Griseofulvin (*Penicillium griseofulvum*), Bacitracin (*Bacillus subtilis*), Polymyxin B (*Bacillus polymyxa*), Amphotericin B (*Streptomyces nodosus*), Erythromycin (*Streptomyces erythreus*), Neomycin (*Streptomyces fradiae*), Streptomycin (*Streptomyces griseus*), Tetracycline

(*Streptomyces rimosus*), Vancomycin (*Streptomyces orientalis*), Gentamicin (*Micromonospora purpurea*), Rifamycin (*Streptomyces mediterranei*) etc. Fungi also have yielded few useful antibiotics. Apart from penicillin, the most important antibiotics from fungi are the cephalosporins (beta-lactams with similar mode of action to penicillin, but with less allergenicity) and griseofulvin (from *Penicillium griseofulvum* and related species), which is used to treat althlete's foot and related fungal infections of the skin.

Fungi have proven to be a very rewarding source of bioactive and structurally diverse natural products (Dreyfuss and Chapela, 1994a). A variety of fungi and their natural beauty occupy prime place in the biological world, and India has been the cradle for a large number of such fungi. Only a fraction of total fungal wealth has been subjected to scientific scrutiny, and mycologists have yet to unravel the unexplored and hidden bio-resource. Fungi are not only the interesting class of organisms but play a significant role in the daily life of human beings besides their utilization in industry, agriculture, medicine, food industry, textiles, bioremediation, natural cycling, as biofertilizers and many other ways (Manoharachary *et al.,* 2005). Microorganisms seem to occupy virtually every living and non-living niche on earth. This includes those in the thermal vents, in deep rock sediments, and in desert as well as marine environments. In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of unimaginable numbers of organisms identified as endophytes (Bacon and White, 2000). Endophytes are the microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation by their presence and have typically gone unnoticed (Strobel and Long, 1998). Fungal endophytes are microfungi that colonize living tissues of plants without producing any apparent symptoms or obvious negative effects (Hirsch and Braun, 1992). Fungi that are biotrophic mutualists, benign commensals or latent pathogens are included under the broad term 'endophytes' (Stone *et al.,* 2000). Many endophytes produce unusual secondary metabolites of industrial importance (Hawksworth *et al.,* 1995; Pirozynski and Hawksworth, 1988). Furthermore, some endophytes are known to contribute to the fitness of their hosts (Manoharachary, 1991; Pirozynski and Hawksworth, 1988; Manoharachary, 1981; Stone *et al.,* 2000; Tan and Zou,

2001; Schulz *et al.,* 2002; Carroll, 1986; Johnson, 1994). As a result of these long-held associations, it is possible to imagine that some of these endophytic microbes may have devised genetic systems allowing for the transfer of information between themselves and the higher plants and vice versa (Stierle *et al.*, 1993). The symbiosis between plant and endophyte has been ascertained, namely, the former protects and feeds the latter, which produces 'in return' bioactive (plant growth regulatory, anti-bacterial, anti-fungal, anti-viral, insecticidal, etc.) substances to enhance the growth and competitiveness of the host in nature (Carroll, 1988). A world-wide scientific effort to isolate endophytes and study their secondary metabolism is now under way. In fact, a comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown (Schutz, 2001).

Camptothecin (CPT), a pentacyclic quinoline alkaloid (Fig.1a), belongs to a group of antineoplastic agents with a unique mechanism of action involving interference with eukaryotic DNA. (Kauh and Bjornsti, 1995; Potmesil and Pinedo, 1995a; Sawada *et al.*, 1995; Torck and Pinkas, 1996). Moreover, one of the primary cellular responses to its exposure is a rapid cessation of RNA synthesis. (Bendixen *et al.,* 1990). This alkaloid displays a unique mechanism of action by inhibiting the intranuclear enzyme topoisomerase I, which is required for the swivelling and relaxation of DNA during molecular events, such as DNA replication and transcription. (Meng *et al.*, 2003). The drug is already used in China for the treatment of skin diseases (Cai and Hutchinson, 1983). Hycamtin (topotecan) and Camptosar (irinotecan), the semi-synthetic derivatives of camptothecin, have been employed clinically for the treatment of ovarian and colon cancers. (Oberlies and Kroll, 2004; Cragg and Newman, 2004). It is also used as an insect chemosterilant, a plant regulator, and an inhibitor of the herpes virus. (Becker and Olshevsky, 1973). In addition, camptothecin prevents the replication of the influenza virus (Kelly *et al.*, 1974). Camptothecin and minor camptothecinoids have been obtained in high yield from the Indian tree *Nothapodytes foetida* (Wight) Sleumer (formerly *Mappia foetida*, Icacinaceae), (Pirillo *et al.*, 1995; Govindachari and Viswanathan, 1972a; Govindachari *et al.*, 1974), commonly known in India as "Kalgur". This small tree is distributed in the western part of peninsular coastal India from Konkan

Ghats to northern parts of the Kanara, Niligiris, Anamalis and Pullneys hills. CPT has also been reported to be present in various Japanese plant species, including *N. collina, N. obscura, N. obtusifolia, N. piltosporsides*, and *N. tomentosa*. (Zhang and Bao, 1990).

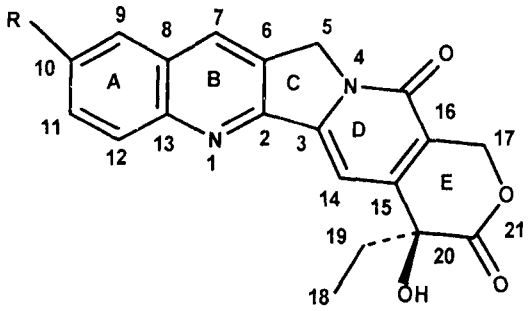

Fig. 1: (a). (+)-Camptothecin, R=H (b). 9-Methoxycamptothecin, R=OMe

The present study includes the isolation of camptothecin and 9-methoxycamptothecin (Fig. 1 a, b) from the aerial parts of *Nothapodytes foetida* by semi-preparative HPLC (Puri *et al.*, 2005a) as these compounds were required as reference compounds for qualitative as well as quantitative analysis. The purity of the isolated molecules was determined by physico-chemical spectral data and LC-MS/MS. Earlier reported procedures for isolation of 9-methoxycamptothecin from the crude extract involved cumbersome procedures encompassing high-speed counter current chromatography and repeated crystallizations (Govindachari and Vishwanathan, 1972a; Broglia *et al.*, 1994). In the present methodology, 9-methoxy-camptothecin has been isolated in 95% purity by semi-preparative HPLC from the mother liquor after crystallization of camptothecin (>95% pure) from the crude extract of *N. foetida*.

Although CPT is a promising anti-tumor agent, it is unfortunately only available in relatively insufficient concentration in the tree roots, which demands the uprooting of rare, 50 to 75 year old trees from the forests. The supplies of CPT available from inconsistent wild sources are, therefore, inadequate when compared to its projected demand. Although synthesis route is reported for CPT, the yield after a multi-steps procedure is low and therefore, of no commercial significance (Wall *et al.*, 1993; Rao *et al.*, 1994; Fang *et al.*, 1994). It is, thus, essential to understand how, where and when CPT is biosynthesized in the plants and to

evaluate the factors that affect its biosynthesis. Besides, it is important to search for alternative natural sources of this important compound. A sustained search in this direction led to isolation of an endophyte from *N. foetida* plant. The organism on extensive qualitative screening was found to accumulate CPT.

In the present study, the isolation of an endophytic fungus, which was identified as *Entrophospora infrequens*-RJMEF001, from *Nothapodytes foetida* that specifically produces camptothecin has been demonstrated. The isolated endophyte was grown on various economically viable, simple and synthetic media to optimize the best nutrients combination for biomass accumulation and CPT production. Growth and production under submerged and surface culture conditions were standardized and chemical characterization of CPT was performed using modern chromatographic and spectroscopic methods. Spectral data, obtained using HPLC, LC-MS, MS/MS and ^{1}HNMR, of the fungal secondary metabolite is identical to authentic molecule. Furthermore, CPT isolated from this source has been found to be biologically active i.e. possesses cytotoxic property against human cancer cell lines. Besides, the fermentation conditions were optimized for up scaling of biomass and secondary metabolite (CPT) accumulation in bioreactor. Furthermore, preliminary surface, solid-state fermentation and precursor studies were also conducted. In addition to the isolation of CPT, the endophyte was also screened for other major and minor chemical constituents. All the minor metabolites were characterized by ^{1}HNMR, MS^{2} and with reference to certain compounds ^{13}CNMR was also used as tool for the assignment of structures. Bioguided fractionation of extracts lead to isolation of three new and two known compounds viz., 4-(3,4-dimethoxy-phenyl-1-oxy)-caffeic acid; 4-(3,4-dihydroxy-phenyl-1-oxy)-ferulic acid; 4[4-(3,4-di-hydroxyphenyl-oxy)-peroxy-feruly]-3-methoxy-salicylic acid; 5-(hydroxymethyl)-2-furfuraldeyde and ergosterol. This study clearly demonstrated that the isolated endophyte may be a potential organism for further development as a source of CPT by fermentation. The organism may also be considered for further studies for unraveling the secondary metabolic pathways leading to CPT and other analogues production.

CHAPTER 2

REVIEW OF LITERATURE

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity and have a minor environmental impact. This search is driven by the development of resistance in infectious microorganisms (e.g., species of *Staphylococcus*, *Mycobacterium*, and *Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms. The ingress to the human population of new diseases such as AIDS and severe acute respiratory syndrome requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but so do new therapies for treating ancillary infections, which are a consequence of a weakened immune system. Furthermore, others who are immunocompromised (e.g., cancer and organ transplant patients) are at a risk for opportunistic pathogens, such as *Aspergillus* spp., *Cryptococcus* spp. and *Candida* spp., that normally are not major problems in the human population. In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections, such as malaria, leishmaniasis, trypanomiasis and filariasis. Malaria alone is more destructive in claiming lives each year than any other single infectious agent with the exception of the AIDS virus and *Mycobacterium tuberculosis* (National Institutes of Health, 2001). Finally, because of safety and environmental problems, many synthetic agricultural agents have been and currently are being targeted for removal from the market, which creates a need to find alternative ways to control farm pests and pathogens (Demain, 2000). Novel natural products and the organisms that make them offer opportunities for innovation in drug and agrochemical discovery.

Background information and discovery of endophytes

Fungi have existed for over 900 million years based on fossil calibrated molecular clock estimations (Berbee and Taylor, 2001; Blackwell, 2000; Heckman *et al.*, 2001). These organisms are adapted to a wide variety of habitats and have made a great impact on all ecosystems (Alexopoulos *et al.*, 1996). The term endophyte originated with De Bary (1866) who used the term to distinguish fungi that reside within host tissues from epiphytes, fungi that live on the outer surfaces of host plants. In the 1980's, the term became restricted to include only those organisms

that cause asymptomatic infections in host and excluded the pathogenic fungi and mutualists such as mycorrhizae (Carroll, 1986).

Since the discovery of endophytes at Darnel in Germany, in 1904 (Tan and Zou, 2001), various investigators have defined endophytes in different ways, which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. Bacon and White gave an inclusive and widely accepted definition of endophytes—"microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Bacon and White, 2000). While the symptom less nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggest that they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. At times, it appears that other microbial forms, e.g., mycoplasmas and archaebacteria, most certainly exist in plants as endophytes, but no evidence for them has yet been presented. So far, the fungi form the bulk of isolated endophytes. According to Hawksworth and Rossman (1987) there may be as many as 1 million different fungal species, yet only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. Dreyfuss and Chapela estimate that there may be at least 1 million species of endophytic fungi alone (Dreyfuss and Chapela, 1994b). It is now obvious that endophytes are going to be one of the richest and reliable sources of genetic diversity and novel, undescribed species. Interestingly, novel microbes usually have associated with them novel natural products.

Endophytes

An endophyte may be a bacterial (including actinomycete) or fungal microorganism, which spends the whole or part of its life-cycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plant typically causing no apparent symptoms of disease (Sturz *et al.*, 2000; Wilson, 1995a). The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis (Strobel and Long, 1998).

Fungal endophytes

Endophytic fungi are an important and relatively less studied group of microbial plant symbionts. Endophytic fungi live asymptomatically, and sometimes systemically, within plant tissues (Carroll, 1988, 1991). Endophytes usually inhabit above-ground plant tissues (leaves, stems, bark, petioles and reproductive structures), which distinguishes them from better known mycorrhizal symbionts. The distinction is not firm, because endophytes may also inhabit root tissues. Overall, endophytic fungi are ubiquitous and extremely diverse in host plants. Every plant examined todate harbors at least one species of endophytic fungus and many plants, especially woody plants, may contain literally hundreds or thousands of species (Petrini, 1986; Petrini *et al.*,1992a; Gaylord *et al.*,1996; Faeth and Hammon, 1997; Saikkonen *et al.*,1998; Arnold *et al.*, 2000).

Why endophytes have been chosen?

There are approximately 300,000 different plant species on our planet. Out of the several hundred of these that have been examined, each one has a complement of endophytic microbes. The plants growing in unique environmental settings, having ethnobotanical uses, having extreme age or interesting endemic locations generally produces novel endophytic micro-organisms. Overall, our rationale for studying endophytic microbes as potential sources of rare, novel and new bioactive molecules is to the fact that this is a relatively unexplored area of biochemical diversity. Furthermore, our search is driven by the fact that the contribution of the endophytes to the plant may be to provide protection to it by virtue of anti-microbial compounds that it produces. Some of these compounds may be of interest to medical sciences, since they possess anti-fungal, anti-bacterial, anti-malarial, and a host of other biological activities. Finally, of major concern to the medical community is the latent toxicity of any prospective drug to the higher organisms such as animal and human tissues. It would appear that since the plant is also a eukaryotic system, in which the endophyte exists, the metabolites made by the endophyte may have reduced cell toxicity; otherwise, death of the host tissue may occur. Thus, the plant itself has naturally served as a selection system for microbes

having bioactive molecules with reduced toxicity toward higher organisms. The prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans besides diseases like cancer, HIV, etc, which take a heavy toll of human beings, plants, and animals are great.

In this review, few representative groups of fungal endophytes have been discussed briefly to highlight the extent of diversity, their associated secondary metabolites and a chemical and biological profile of some important leads.

Biological survey of endophytes

Almost all vascular plant species examined todate were found to harbor endophytic bacteria and/or fungi. (Sturz *et al.,* 2000; Arnold *et al.,* 2000). Moreover, the colonization of endophytes in marine algae, (Smith *et al.,* 1989) mosses and ferns (Petrini, 1991; Raviraja *et al.,* 1996) has also been recorded. As a matter of fact, endophytes are important components of microbial biodiversity (Clay, 1992). Commonly, several to hundreds of endophyte species can be isolated from a single plant, among them, at least one species showing host specificity. The environmental conditions under which the host is growing also affect the endophyte population, (Hata *et al.,* 1998) and the endophyte profile may be more diversified in tropical areas. Moreover, genotypic diversity has been observed in single endophyte species originating from conifers, (Leuchtmann *et al.,* 1992; Mc Cutcheon *et al.,* 1993) birch (Lappalainen and Yli-Mattila, 1999) and grasses (Reddy *et al.,* 1998). Accordingly, endophytes are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location.

Origin and evolution

Some phytopathogens in the environment are of endophytic origin (Carroll, 1988). Many innocuous fungal endophytes are quiescent phytopathogens, which may cause infectious symptoms when the host plant is aged and/or stressed. On the other hand, during the long co-evolution of the phytopathogen and its host plant, an endophytic mutant may result from balanced antagonism and/or gene mutation. Dual cultures of the host calli and endophytes demonstrated that both the endophytes and the host calli excrete metabolites toxic to each other (Sieber *et al.,*

1990; Peters *et al.*, 1998). Further investigations led to the development of a hypothesis that the endophyte–host interaction could be a balanced pathogen–host antagonism (Schulz *et al.*, 1999). Freeman and Rodriguez (1993) found that a naturally occurring non-pathogenic endophytic mutant developed from the mutation of a single locus in the genome of the wild-type *Colletotrichum magna*, a pathogen causing anthracnose in cucurbit plants. This mutant is able to grow systemically inside the host plant without pathogenic symptoms, but retaining wild-type levels of *in vitro* sporulation, spore adhesion, appressoria formation, infection and host specificity. The *Acremonium* (asexual fungi now reclassified in the genus *Neotyphodium*, Glenn *et al.*, 1996) endophytes, which usually inhabit tall fescue, perennial ryegrass (*Lolium perenne* L.), and many cool-season grasses, are considered mutualistic symbionts of the host grasses. The grass and the endophytic fungus are so intimately associated that they act as a whole, much like 'a single organism'. And, indeed, some of these endophytic *Neotyphodium* species can only spread by infecting seeds from the mother plants (Schardl and Philips, 1997).

Host-Endophyte interactions

Mutualistic associations

Like mycorrhizae, endophytic fungi are thought to interact mutualistically with their host plants mainly by increasing host resistance to herbivores (Carroll, 1988, 1991; Clay, 1988, 1990) and have been termed "acquired plant defenses" (Cheplick and Clay, 1988). Indeed, some agronomic grass species infected with systemic endophytes show striking toxic and noxious effects on vertebrate and invertebrate herbivores (Clay, 1988, 1990, 1992; Siegel and Latch, 1987; Breen, 1994) and pathogens (Gwinn and Gavin, 1992) by virtue of alkaloids such as pyrrolizidine alkaloids, ergot alkaloids and peramine produced by the fungi (Powell and Petroski, 1992; Siegel and Bush, 1996; Leuchtmann *et al.*,2000). Endophytes, at least systemic ones in agronomic grasses, may also increase host grass competitive abilities, by increasing germination success, resistance to drought and water stress and resistance to seed predators (Clay, 1988; Wolock-Madej and Clay, 1991; Knoch *et al.*,1993). In return, plants provide spatial structure and protection from

desiccation, nutrients and photosynthate and, in the case of vertical-transmission, dissemination to the next generation of hosts.

Alternatively, systemic grass endophytes, at least in some introduced agronomic grasses, as well as a few native grasses (Faeth and Bultman, 2002) may have profound effects on herbivores. *Epichloe* and *Neotyphodium* endophytes in these introduced grasses cause toxicosis to grazing livestock (Clay, 1990, 1991, 1992; Hoveland, 1993), and increase resistance to invertebrate herbivores and pathogenic microorganisms (Carroll, 1988; Clay, 1988, 1990; Clay *et al.*,1993; Dahlman *et al.*,1991; Kimmons *et al.*,1990; West *et al.*,1993; Breen, 1994) and their natural enemies (Bultman *et al.*,1997; Ormacini *et al.*, 2000) and may inhibit germination and growth of other grasses via allelopathy by endophyte alkaloids (Peters and Zam, 1981; Petroski *et al.*,1990). Neotyphodium-linked alkaloids (ergot and indole diterpene type alkaloids) produce "staggers" (a neurological disorder) in sheep and cattle, while in tall fescue, pyrrolizidine and ergot-type alkaloids cause gangrene of extremities, reduced conception and generally poor health in livestock (Siegel and Bush, 1996; Schardl and Phillips, 1997). Resistance to insect pests in infected tall fescue and perennial ryegrass is mainly the result of peramine and pyrrolizidine alkaloids in tall fescue and ryegrass (Breen, 1994; Siegel and Bush, 1996). While endophytes may confer other benefits to their hosts, such as increased drought resistance (Richardson *et al.*,1992, 1993; West *et al.*,1993; Arechavaleta *et al.*,1992; Bacon, 1993), alkaloids produced by symbiotic endophytes mediate many known benefits (Siegel and Bush, 1996).

Resistance to diseases

Phytoalexin production by the host plant in reaction to infection by endophyte can actually render the host resistant to attack by pathogens (Wilson, 1993). The absence of endophytes in glasshouse-raised plants may therefore explain their acute susceptibility to insect and fungal pests and diseases, since these plants are protected against natural air borne inoculum of endophytes (Wilson, 1993). Mutual exclusion of endophytes within leaves where infection by one species may inhibit infection by another is also documented. For example, leaves sprayed with *Asteromella* sp. or *Plectophomella* sp., which are recognized endophytic fungi,

were able to exclude other endophytic fungal infectons (Wilson, 1996). *Cryptosporiopsis abietina* is a stem endophyte of *Picea sitchensis,* and shows antagonistic activity against *Heterobasidion annosum.* The fungus also behaves as an aggressive seedling pathogen on *Picea abies* and can be associated with declining mycorrhizae (Holdenrieder and Sieber, 1992). Bissegger and Sieber (1994) also isolated from European chestnut a fungus with antifungal properties, related to crytosporiopsis, namely *Pezicula cinnamomea.* Sacco *Pezicula cinnamomea* inhibited other pathogens, including *Cryphonectria parasitica* (Murrill) Barr in dual cultures, possibly rendering it as an effective natural bio-control agent (Bissegger and Sieber, 1994). Due to the fungitoxic effects the *Balansia cyperi* Edgerton, an endophyte of *Cypems rotundus* L., fungus is able to exclude pathogens such as *Rhizoctonia solani* Kuhn, from the leaves of its host (Stovall and Clay, 1991). *In vitro* bioassays with mycelium and culture filtrates of *B. cyperi* showed inhibition of test fungi, which included *Fusarium oxysporum* Schlechtend and *R solani.* Solvent extracts made of leaves from *B.cyperi*-infected plants, also inhibited the growth of fungi including *F. oxyspomm, Rhizoctonia oryzae* Ryker and Gooch and *R. solani.* These results show the ability of *B. cyperi* to prevent infection of *C. rotllndus by* other pathogenic fungi (Stovall and Clay, 1991).

Secondary metabolites produced by fungal endophytes in tomato roots are highly toxic to *Meloidogyne incognita,* especially strains of *F. oxyporum* (Hallmann and Sikora, 1996). These toxins were produced by a non-pathogenic strain of *F.oxysporum* and were highly effective towards sedentary parasites, less effective towards migratory endoparasites, and non-parasitic nematodes were not influenced at all. Metabolites of this fungus (*F.oxysporum*) also reduced the growth of pathogens such as *Phytophthora cactorum* (Lebert and Cohn) J. Schrot., *Pythium ultimum* Trow and *Rhizoctonia solani* in *in vitro* studies (Hallmann and Sikora, 1996). Biological control of certain diseases, such as chestnut blight caused by *Cryphonectria parasitica* on *Castanea sativa,* can be obtained by spreading hypovirulence by means of endophytic thalli from hypovirulent strains of *Cryphonectria parasitica* (Bissegger and Sieber, 1994).

Protection from insect herbivory

It is generally believed that endophytic fungi can affect the interaction between their hosts and insect herbivores. Where a mutualistic association exists between fungi and insects, it will result in increased herbivory of host plants, and a mutualistic association between fungi and plants, in reduced herbivory of the host plant (Clay, 1987). When the endophyte-plant symbiosis is strongly mutualistic and the host benefits through increased defence against herbivores, the host may rely largely or wholly on the endophytes for their resistance (Wilson, 1993). Systemically infected grasses display an increased level of resistance to a wide variety of insect and mammalian herbivores as a result of alkaloids produced by fungi (Clay, 1987). Certain endophyte species inhabiting conifer needles produce compounds that could be linked to the mortality or decreased growth of spruce budworm larvae (Clark *et al.,* 1989). Some species are in the genus *Leptostroma,* but the most toxic strains are not yet identified and could represent new genera. These coniferous endophytes produce compounds that affect spruce budworm, mortality, or retard larval development (Clark *et al.,* 1989). Calhoun *et al.*, (1992) identified four toxic metabolites produced by endophytes of balsam fir, which are effective against spruce budworm. *Phyllosticta sp.* produced heptelidic acid, heptelidic acid chlorodydrin and hydroheptelidic acid. A compound, (+)-rugulosin, an anthraquinone was produced by *Hornonema dematioids* and exhibits a wide spectrum of biological activity.

The most important endophyte of Douglas fir, *Meria parkeri* Sherwood- Pike, produces compounds that are toxic to insects (Todd, 1988). Gange, (1996) reported that the larvae of the pine processionary moth *(Thaumetopoea pityocampa)* avoided endophyte-infected needles of *Pinus bmtia,* Insect death can also be attributed to starvation in the case *Quercus garryana,* where the endophytic fungus kills the galls of a cynipid wasp, and deprives the insects of food (Wilson, 1995b). A strain of endophytic *Pencillium* sp., which might produce rotenone or its analogues and showed bioactivity against aphids, was isolated from the fresh roots of *Derris elliptica* Benth. (Hu *et al.,* 2005).

Growth promotion of the host plant

Some endopytes promote growth of their host plants. *Leptodontium orchidicola* Sigler and Currah, a dematiacious hyphomycete isolated from roots of subalpine plants, caused a significant increase in host root length of *Salix glauca* L. seedlings, but the fungus also invaded the stele, causing extensive cellular lysis (Fernando and Currah, 1996). *Phialocephala fortinii* Wang and Wilcox has an amensal, parasitic or neutral association with its host and, in combination with *Potentilla fruticosa* L., results in a significant increase in shoot weight (Fernando and Currah, 1996). Root chemical characteristics that facilitate colonization of internal root tissues with subsequent growth promoting possibilities for the host plant (Shishido *et al.,* 1995). Endophyte-infected plants often grow faster than non-infected ones (Cheplick *et al.,* 1989). This effect is at least in part due to the endophytes' production of phytohormones such as indole-3-acetic acid (IAA), cytokines and other plant growth-promoting substances, and/or partly owing to the fact that endophytes could have enhanced the hosts' uptake of nutritional elements such as nitrogen (Resis *et al.,* 2000) and phosphorus. (Gasoni and Gurfinkel, 1997; Malinowski and Belesky, 1999).

Detrimental endophytic associations

However, the beneficial effects of endophytes, especially those related to herbivory, are much less clear in native grasses. For example, *Neotyphodium* infections in most native grasses are not toxic to livestock and other vertebrates (Schulthess and Faeth, 1998; Saikkonen *et al.,*1998; Faeth, *et al.*, 2002; Faeth and Bultman, 2002) or invertebrates (Lopez *et al.,*1995; Tibbets and Faeth, 1999). However, more recent arguments and evidence suggest that interactions between host plants and endophytes are not fixed in either ecological or evolutionary time, or geographically (Saikkonen *et al.*, 1998; Faeth and Bultman, 2002) and range from mutualistic to antagonistic. This view is in keeping with more recent and general concepts of species interactions, and mutualisms in particular (Law, 1985; Lewis, 1985; Carroll, 1992; Connor, 1995; Thompson, 1982, 1994, 1999; Pellmyr and Thompson, 1992; Pellmyr *et al.,* 1996; Saikkonen *et al.,* 1998; Morris, 1996). For example, many plant-mycorrhizal interactions, the belowground counterparts

of endophytes, are now recognized as ranging from mutualistic to antagonistic, depending on phylogeny, genetic strains, other interacting species, geography and abiotic conditions (Parker, 1995, 1999; Johnson, 1993; Johnson *et al.*, 1997; Gehring and Whitham, 1994; Gehring *et al.*, 1997).

Latent pathogenesis

Plant pathologists rigidly following Koch's postulates, have discarded latent pathogens as 'saprophytes' or 'secondary parasities', since no symptoms were detected following inoculation of a vigorous host. Alternatively, they have labeled latent pathogens as aggressive pathogens without considering possible predisposing factors (Schoeneweiss, 1975). A parasitic relationship usually starts when the infection hypha of a fungus penetrates the host cuticle and then the outer epidermal cell wall (Verhoef, 1974). In some instances, however, some time may pass between penetration and the start of such a parastic relationship, which is then referred to as a latent, dormant, or quiescent infection (Verhoef, 1974). The latent period is defined as the time from infection until the expression of macroscopic symptoms, or as prolonged incubation period (Sinclair and Cerkauskas, 1996). Only fungi colonizing living tissue can potentially be termed latent pathogens (Kowalski and Kehr, 1996).

Latent-infecting fungi as well as endophytes can infect plant tissues and become established after penetration, but infection does not imply the production of visible disease symptoms. According to Sinclair and Cerkauskas (1996), latent infection of plants by pathogenic fungi is considered one of the highest levels of parasitism.

Comparative studies by Espinosa-Garcia and Langenheim (1991), on the effect of essential oils on three pathogenic and one endophytic fungus demonstrated differences in tolerance to essential oils between pathogens and endophytes. The relatively high tolerance showed by the pathogens, *Phomopsis occulta* (Sacc.) *Traverso*, *Pestalotiopsis funereal* and *Seiridium juniperi* (Allesch.) Sutton to essential oil phenotypes of redwood, reflect their adaptation to the host defence reactions that involve terpenoids. The coniferous endophyte, *Cryptosporiopsis abientina*, on the other hand, displayed and overall susceptibility to the redwood essential oils (Espinosa Garcia and Langenheium, 1991).

Symptom expression elicited by environmental stress

A significant number of endophytic fungi in healthy plants become pathogenic when their host plants are weakened. In this instance the host-fungus interaction manifests itself as a disease syndrome (Dorworth and Callan, 1996). These mutualistic modes change to a necrotrophic mode when the host plant is predisposed by several factors such as stress (Schoeneweiss, 1975). Latent infections by endopytes do not result in the formation of disease symptoms, but may weaken the plant predisposing it to other stresses or diseases (Sinclair and Cerkauskas, 1996). Endophytes and weak parasites of Quercus, namely *Pezicula cinnamomea* and *Colpoma quercinum*, may contribute to the death of weakened tissue, but the aggressive *Fusicoccum quercus Oud.*, causal agent of annual canker is hardly ever isolated as an endophyte (Kowalski and Kehr, 1996).

Kowalski (1993) isolated the pathogen of autumn needle cast, *Cyclaneusma minus* twice as frequently from symptomless needles of trees that showed symptoms of second year needle cast, than from trees without such symptoms. Tree showing needle cast symptoms had an overall higher susceptibility to fungal infection already on their first year needles (Kowalski, 1993). It is well known that plant pathogenic fungi express an incubation phase before disease symptoms appear. In the case of *Cyclaneusma minus*, this latent phase extends more than 15 months, which might explain its 'endophytic' nature and high colonization frequency in pine needles (Kowalski, 1993).

Cenangium ferruginosum is documented as a pathogen causing shoot dieback of pines, but it also seems to live as an endophyte in the needles of *Pinus sylvestris* (Kowalski, 1993). Wood decay of dying trees possibly originates from infections of latent fungi present in healthy, living branches (Chapela and Boddy, 1988a). These fungi are in a state of environmental conditions which include the reduction of water content in the xylem of the tree. The variation in endophytic colonization between annual rings could be attributed to variation in tree susceptibility and inoculum potential (Chapela and Boddy, 1988b). *Botryosphaeria dothidea* (Moug.) Ces. Et de Not. is the causal agent of die-back, canker and leaf spots of Eucalyptus *spp.* in South Africa, but it is also able to colonize the xylem and leaves of trees asymptomatically (Smith *et al.*, 1996a). Disease symptoms develop

rapidly at the onset of environmental stress such as frost, hot winds or drought, which can be seen as the trigger for the pathogenic stage of the pathogen (Smith *et al.*, 1996b).

Indirect enhancement of insect colonization and inhibition of host plant growth

The endophyte, *R. parkeri*, may slightly inhibit the growth of its host, Douglas fir at high levels of infection, but has no other deleterious effect on the growth of the host (Todd, 1988). On the other hand, some endophytes can actually have a positive effect on insect colonization. Gange (1996) proved that infection of Sycamore (*Acer pseudoplatanus* L.) leaves by an endophytic fungus, *Rhytisma acerinum* (Pers.) Fries, positively affected the number of aphids (*Drepanosiphum platanoides* (Schr.) and *Periphyllus acericola* (Walk.) on leaves, especially during summer. This could possibly be attributed to the higher amount of soluble and total nitrogen, and totaled carbon contents of infected leaves. It is possible that the digestive processes of the fungus alter total carbon or nitrogen contents as compounds are moved into or out of leaves by the host, in this way altering the food quality of these tissues. The presence of endophytes may therefore also determine the seasonal patterns of herbivory by these aphids (Gange, 1996).

Utilization and Manipulation of Endophytic Associations

Bio-control of weeds

Until now the only recognized means of controlling weeds killing or constraining growth of newly planted forest trees were to use chemical herbicides or by controlled burning. Both of these methods have attracted huge criticism from environmental groups and thus other means of control have to be investigated (Dorworth and Callan, 1996). Some endophytic fungi show promise as second order (II^{o}) biocontrol agents of forest weeds, but first order biocontrol (I^{o}) does not involve endophytes. Research on biocontrol through the application of endophytes has the goal of promoting internal fungi from resident to necrotrophic status by stimulating the fungi themselves or by reducing the physiological vigour of the host plant, or reaching a suitable combination of the two. Endophytes themselves may

also predispose their hosts to environmental damage by reducing the damage threshold (Dorworth and Callan, 1996).

Bio-control of other pathogens

An endophytic *Cryptosporiopsis sp.* isolated from *Vaccinium myrtillus* L. produced three different antibiotic-containing substances, which are all inhibitory of *Candida albicans* (C.P. Robin) Berkhout, a common human pathogen (Fisher *et al.,* 1984). Noble *et al.,* (1991) isolated and identified an echinocandin from an endophytic *Cryptosporiopsis sp.* derived from twigs of *P. sylvestris*, and a *Pezicula* sp. derived from twigs of *Fagus sylvatica.* This compound proved to have antimicrobial properties against certain yeasts. Fungi which produce such potent antifungal properties give them a competitive advantage over other potential fungal colonizers (Noble *et al.,* 1991).

Improvement of the hosts' ecological adaptability

Certain endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on the host plant. Endophyte-infected grasses usually possess an increased tolerance to drought (Arachevaleta *et al.,* 1989; Ravel *et al.,* 1997) and aluminium toxicity. (Malinowski and Belesky, 1999) Furthermore, some endophytes are able to provide the host plant with protection against some nematodes, (Kimmons *et al.,* 1990; Hallmann and Sikora, 1996) mammal (Bacon *et al.,* 1977) and insect herbivores (Preszler *et al.,* 1996; Wilkinson *et al.,* 2000) as well as bacterial and fungal pathogens. (Christensen, 1996; Sturz *et al.,* 1999) Some endophytes are capable of enhancing the hosts' allelopathic effects on other species co-growing nearby, usually being competitor(s) for the nutrition and the space. (Sturz and Christie, 1996; Sturz *et al.,* 1998; Sutherland *et al.,* 1999). This could be the reason why some plants with special endophytes are usually competitive enough to become dominant species in successional fields (Clay and Holah, 1999).

Diversity spectrum of fungi

The number of fungi recorded in India exceeds 27,000 species and form the largest biotic community after insects (Sarbhoy *et al.,* 1996). The true fungi belong to kingdom Eukaryota which has four phyla, 103 orders, 484 families and 4979 genera. The eighth edition of *Dictionary of the Fungi* (Hawksworth *et al.,* 1995) has recognized eleven phyla. The Deuteromycotina is not accepted as a formal taxonomic category. The number of fungal genera reported from the world and that from India between 1905 and 1995, are shown in Table 1. About 205 new genera have been described from India, of which 32% were discovered by C. V. Subramanian of the University of Madras. Of these, approximately 27,000 species are reported to colonize diversified habitats (Sarbhoy *et al.,* 1996). This indicates a ten-fold increase in the last 70 years. Manoharachary and his co-workers (Manoharachary, 2001) have added 12 new genera, 60 new taxa and 500 new additions to fungi of India. The fossil record of fungi dates back to the early phanerozoic and into the proterozic geological era (Pirozynski and Hawksworth, 1988). The existence of fossil fungi indicates their evolutionary significance besides solving certain phylogenetic complexities.

Endophytes and Biodiversity

Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and the most biodiverse microorganisms. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor more than 60% of the world's terrestrial biodiversity (Mittermeier *et al.*, 1999). As such, one would expect that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is the most active. Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and

Table 1: Fungal genera

Phyla	World	India
Myxomycotina	450	380
Mastigomycotina	308	205
Zygomycotina	55	50
Ascomycotina	2000	745
Basidiomycotina	357	232
Deuteromycotina	4100	468
Total	**7270**	**2080**

biologically active compounds (Redell and Gordon, 2000). Various authors (Bills *et al.,* 2002) describe a metabolic distinction between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other tropical substrata (Bills *et al.,* 2002). This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

Tropical endophytes and the issue of fungal diversity

Hawksworth (1991) estimated that there are 1.5 million species of fungi; of these, only 75,000 species have been so far described. Several mycologists have tried to answer the question 'Where are the missing fungi?' by identifying the habitats that are to be studied for the presence of such fungi (Dingle and McGee, 2003; Suryanarayanan *et al.,* 2004). The internal tissues of plants harbouring endophytes may well account for a substantial number of new fungi (Dreyfus and Chapela, 1994; Hawksworth, 2001). Tropical plants are expected to support a high diversity of endophytes (Lodge *et al.,* 1996) and only a few of them have been screened for endophyte presence (Rodrigues and Peini, 1997; Suryanarayanan *et al.,* 2001) Arnold *et al.* (2000), based on the results of their study on leaves of two understorey tree species in Panama, suggested that tropical forests are hyperdiverse with reference to endophytes to such an extent that the figure of 1.5 million markedly underestimates fungal diversity. Such an argument is not untenable since the high plant diversity in the tropics is supposed to mirror endophyte diversity. However, some recent studies show that not all tropical forests are as hyperdiverse for endophytes (Cannon and Simmons, 2002). Suryanarayanan *et al.* (2002) studied tropical forests in the Nilgiri Biosphere Reserve of the Western Ghats in India for endophyte assemblages based on host recurrence and spatial heterogeneity of their endophytes and concluded that the dry tropical forests had much less endophyte diversity compared to wet tropical forests. Host specificity of endophytes

concomitant with high host diversity is expected to increase the diversity of endophytes and consequently of fungi in the tropics. However, in their study of 24 trees in the Western Ghats, Suryanarayanan *et al.,* (2003) observed neither host specificity among the endophytes nor association of distinct fungal communities with any host tree. Cannon and Simmons, (2002) obtained similar results for 12 tree species of Iwokrama forest reserve in Guyana. The low endophyte diversity in some tropical forests is attributed to the presence of dominant generalists and low frequency of occurrence of host specific forms among the endophytes (Suryanarayanan *et al.,* 2002; Suryanarayanan *et al.,* 2003). Furthermore, molecular evidence shows that certain fungi, such as *Phyllosticta capitalensis* and *Colletotrichum* spp., have a very wide host and geographical range. For example, *P. capitalensis* occurs as an endophyte in South Africa (Baayen, 2002), Japan, Thailand (Okane *et al.,* 2003), India (Pandey *et al.,* 2003) and Brazil (Rodrigues *et al.,* 2004), which suggests that this fungus could have been described several times as different species, especially since species name in case of the genus *Phyllosticta* is almost invariably based on the host from which it was isolated. This may be true for a few other *Coelomycete* taxa (Rehner and Uecker, 1994). Molecular studies on *Colletotrichum* endophytes isolated from trees of Guyana have also confirmed that at least in some cases fungal diversity may be inversely related to host diversity (Lu, *et al.,* 2004). Recent studies have demonstrated that fungal endophytes are neither passive residents (Sparrow, 1960) nor a mere assemblage of latent pathogens of their hosts (Ganley *et al.,* 2004). They possibly represent a storehouse of new species of fungi, especially in the tropics (Rodrigues *et al.,* 1990; Jacob and Bhat, 2000). Since, only a few plant hosts and habitats have been studied for endophytes, the importance of such studies cannot be overstressed (Suryanarayanan *et al.,* 2001; Sridhar, 2004).

Endophytes and Phytochemistry

The reason why some endophytes produce certain phytochemicals originally characteristic of the host might be related to a genetic recombination of the endophyte with the host that occurs in evolutionary time (Tan and Zou, 2001). This is a concept that was originally proposed as a mechanism to explain why the

endophytic fungus *T. andreanae* may be producing paclitaxel (Stierle *et al.,* 1993). Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce, effectively reducing its market price. All aspects of the biology and interrelatedness of endophytes with their respective hosts is a vastly under investigated inspite of the fact that it is one of the most exciting field. Currently, no one is quite certain of the role of endophytes in nature and what appears to be their relationship to various host plant species. While some endophytic fungi appear to be ubiquitous (e.g., *Fusarium* spp., *Pestalotiopsis* spp., and *Xylaria* spp.), one cannot definitively state that endophytes are truly host specific or even systemic within plants any more than one can assume that their associations with plants are chance encounters. Frequently, many endophytes (biotypes) of the same species are isolated from the same plant and only one of the endophytes will produce a highly biologically active compound in culture (Li, *et al.,* 1996). A great deal of uncertainty also exists between what an endophyte produces in culture and what it may produce in nature. It does seem apparent that the production of certain bioactive compounds by the endophyte *in situ* may facilitate the domination of its biological niche within the plant or even provide protection to the plant from harmful invading pathogens. This may be especially true if the bioactive product of the endophyte is unique to it and is not produced by the host. Seemingly, this would more easily facilitate the study of the role of the endophyte and its role in the plant. Furthermore, little information exists relative to the biochemistry and physiology of the interactions of the endophyte with its host plant. It would seem that many factors changing in the host as related to the season and age, environment, and location may influence the biology of the endophyte.

Physiological and ecological roles

Endophytes colonizing inside plant tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites (Fig.2).

Bioactive molecules from endophytic microbes: A Glimpse

The World Health Organization (WHO) estimates that approximately 80% of the world relies on natural sources directly or indirectly for primary medical treatment and that the health care systems for the remaining 20% of the population also incorporate natural sources in their medical treatment (Cragg, 2002.). In a study of the pharmaceuticals in the market from 1981-2002, only 43% of the drugs were purely synthetic, while remaining 57% were derived from a natural source (Newman *et al.,* 2003). The data shown in the Fig. 3 have been categorized in the following way: biological - a peptide or protein isolated from an organism or cell line; natural product derivative-derived from a natural product usually with some semi-synthetic modifications; synthetic drug; synthetic drug with a natural product pharmacophore; vaccine (Newman *et al.,* 2003).

Some possible sources of natural products include plants, marine organisms, microbes and fungi. Of the approximately 250,000 higher species of plants it is estimated that only 5-15% have been investigated for natural products (Cragg and Newman, 2001a). Also research suggests that less than 1% of bacterial species and less than 5% of fungal are currently known (Cragg and Newman, 2001b).

Endophytes have been recognized as a repository of novel metabolites of pharmaceutical importance (Tan and Zou, 2001).

The following section shows some examples of natural products obtained from endophytic microbes and their potential in the pharmaceutical and agrochemical arenas.

Endophytic microbial products as antibiotics

Most of the antibiotics are defined as low-molecular-weight organic natural products made by microorganisms that are active at low concentration against other microorganisms (Demain, 1981). Often, endophytes are a source of these antibiotics. Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals.

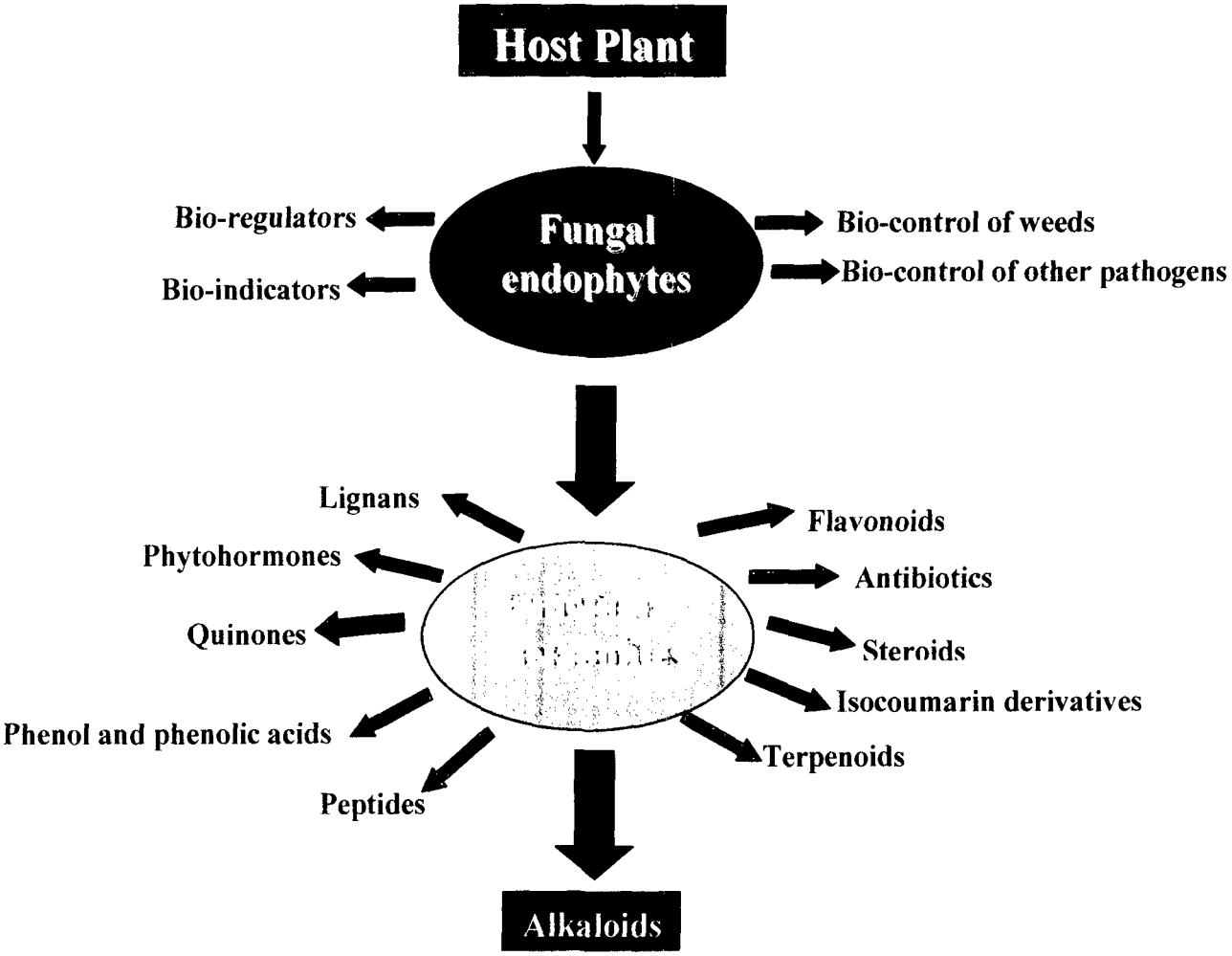

Fig. 2: Host plant- endophyte interactions

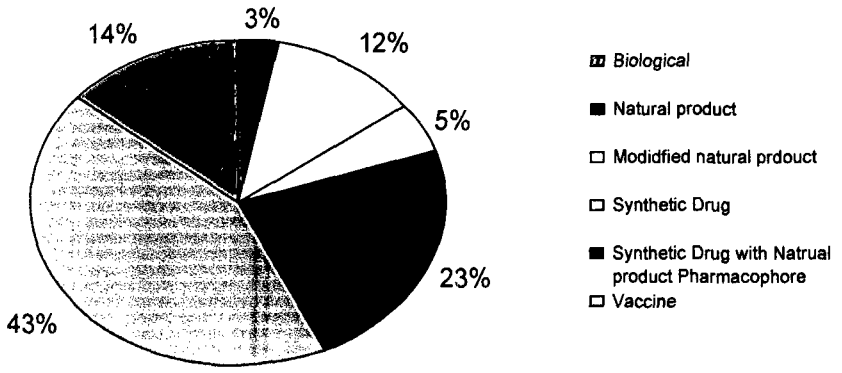

Fig. 3: Pharmaceuticals containing natural products (1981-2002), adapted from Newman *et al.*, (2003)

Cryptosporiopsis quercina is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia (Strobel *et al.*, 1999). On petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens-*Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed cryptocandin, was isolated and characterized from *C. quercina* (Strobel *et al.*, 1999). This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid: 3, 4-dihydroxy methyl proline (Fig. 4). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins (Walsh, 1992). As is generally true not one but several bioactive and related compounds are produced by a microbe. Thus, other antifungal agents related to cryptocandin are also produced by *C. quercina.* Cryptocandin is also active against a number of plant-pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungi causing diseases of skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (Li *et al.*, 2000a) (Fig. 5). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant-pathogenic fungi (Li *et al.*, 2000a). The compound was generally ineffective against a general array of human-pathogenic fungi.

The ecomycins are produced by *Pseudomonas viridiflava* (Miller, 1998). *P. viridiflava* is a member of a group of plant-associated fluorescent bacteria. It is generally associated with the leaves of many grass species and is located on and within the tissues (Miller, 1998.). The ecomycins are active against such human-pathogenic fungi as *Cryptococcus neoformans* and *C. albicans.*

Another group of antifungal compounds is the pseudomycins, produced by a plant-associated pseudomonad (Ballio *et al.*, 1994; Harrison *et al.*, 1991). The pseudomycins represent a family of lipopeptides that are active against a variety of plant- and human-pathogenic fungi. Some of the notable target organisms include *C. albicans*, *C. neoformans*, and a variety of plant-pathogenic fungi, including *Ceratocystis ulmi* (the Dutch elm disease pathogen) and *Mycosphaerella fijiensis*

(the causal agent of Black Sigatoka disease of banana) (Harrison *et al.*, 1991; Strobel, unpublished data). The pseudomycins contain several nontraditional amino acids, including L-chlorothreonine, L-hydroxy aspartic acid, and both D- and L-diaminobutryic acid. The molecules are candidates for use in human medicine especially after structural modification has successfully removed mammalian toxicity (Zhang *et al.*, 2001). Although the pseudomycins are also effective against a number of ascomycetous fungi, they are also being considered for agricultural use.

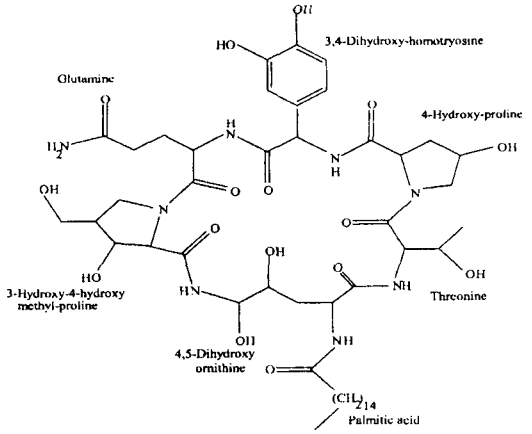

Fig. 4:Cryptocandin A, an antifungal peptide obtained from the endopytic fungus *C. quercina*

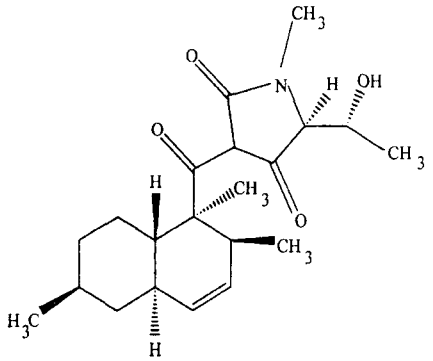

Fig. 5: Cryptocin, a tetramic acid antifungal compound also found in *C. quercina*

Pestalotiopsis microspora is a common rainforest endophyte (Strobel *et al.*, 1996, Strobel, 2002a). It turns out that enormous biochemical diversity does exist in this endophytic fungus, and as such there seem to be many secondary metabolites produced by a myriad of strains of this widely dispersed fungus. One such

secondary metabolite is ambuic acid, an antifungal agent which has been recently described from several isolates of *Pestalotiopsis microspora* found as representative isolates in many of the world's rainforests (Li *et al.*, 2001) (Fig.6). In fact, this compound and another endophyte product, terrein, have been used as models to develop new solid-state nuclear magnetic resonance (NMR) tensor methods to assist in the characterization of molecular stereochemistry of organic molecules (Harper *et al.*, 2001, Harper *et al.*, 2003a).

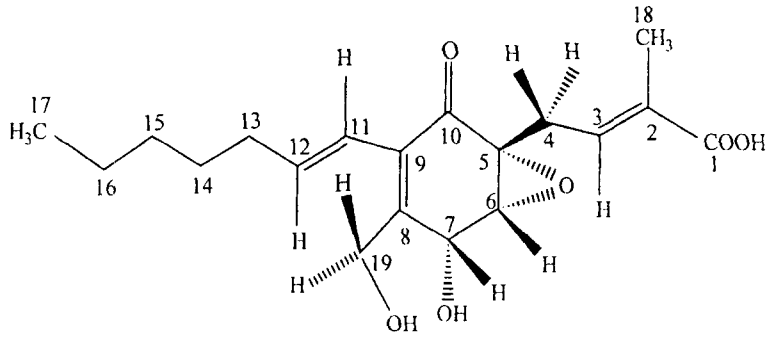

Fig.6: Ambuic acid, a highly functionalized cyclohexenone produced by a number of isolates of *P. microspore* found in rainforests around the world

A strain of *Pestalotiopsis microspora* was also isolated from the endangered tree *Torreya taxifolia* and produces several compounds that have antifungal activity, including pestaloside, an aromatic ß glucoside, and two pyrones: pestalopyrone and hydroxypestalopyrone (Lee *et al.*, 1995a). These products also possess phytotoxic properties. Other isolated secondary products obtained from *Pestalotiopsis microspora* (endophytic on *T. brevifolia*) include two new caryophyllene sesquiterpenes-pestalotiopsins A and B (Pulici *et al.*, 1996a). Other novel sesquiterpenes produced by this fungus are 2-hydroxydimeninol and a highly functionalized humulane (Pulici *et al.*, 1996b, Pulici *et al.*, 1996c). Variation in the amount and kinds of products found in this fungus depends on both the cultural conditions of the organism as well as the original plant source from which it was isolated.

Species of *Pestalotiopsis*, namely, *Pestalotiopsis jesteri*, from the Sepik River area of Papua New Guinea, produces jesterone and hydroxy-jesterone, which exhibit antifungal activity against a variety of plant-pathogenic fungi (Li and Strobel,

2001). Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity (Hu *et al.*, 2001) (Fig. 7).

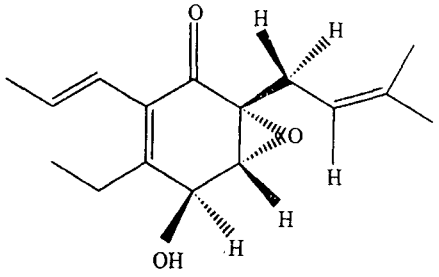

Fig. 7: Jesterone, a cyclohexenone epoxide from *P. jesteri* that has antioomycete activity

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp., represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite mainly exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 µg/disk) against *Bacillus subtilis* (12-mm zone of inhibition), *Salmonella enterica* serovar Gallinarum (11-mm zone of inhibition), and *Staphylococcus aureus* (8-mm zone of inhibition). It also displays a moderate activity against the yeast *Candida tropicalis* (8-mm zone of inhibition) (Horn *et al.*, 1995).

An endophytic *Fusarium* sp. from the plant *Selaginella pallescens*, collected in the Guanacaste Conservation Area of Costa Rica, was screened for antifungal activity. A new pentaketide antifungal agent, CR377, was isolated from the culture broth of the fungus and showed potent activity against *C. albicans* in agar diffusion assays performed on fungal lawns (Brady and Clardy, 2000).

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou *et al.*, 2000). Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed varied antimicrobial activity as well. *A. annua* is a traditional Chinese herb that is well recognized for its synthesis of artemisinin (an antimalarial drug) and its ability to inhabit many geographically different areas. The *Colletotrichum* sp. found in *A. annua* produced not only metabolites with activity against human-

pathogenic fungi and bacteria but also metabolites that were fungistatic to plant-pathogenic fungi (Lu *et al.* 2000).

In addition to plants such as *A. annua* producing antimalarial compounds, some endophytes have shown powerful activity against protozoal diseases as well. Wide-spectrum antibiotics are produced by *Streptomyces* sp. strain NRRL 30562, an endophyte in *K. nigriscans* (Castillo *et al.,* 2002). These antibiotics, called munumbicins, possess widely differing biological activities, depending on the target organism. In general, the munumbicins demonstrate activity against gram-positive bacteria such as *Bacillus anthracis* and multidrug-resistant *M. tuberculosis* as well as a number of other drug-resistant bacteria. However, the most impressive biological activity of any of the munumbicins is that of munumbicin D against the malarial parasite *Plasmodium falciparum*, for which the 50% inhibitory concentration is 4.5 ± 0.07 ng ml^{-1} (Castillo *et al.,* 2002). Another endophytic streptomycete (NRRL 30566), from a fern-leaved *Grevillea* tree (*Grevillea pteridifolia*) growing in the Northern Territory of Australia, produces, in culture, novel antibiotics called kakadumycins (Castillo *et al.,* 2003). Each of these antibiotics contains, by virtue of their amino acid compositions, alanine, serine, and an unknown amino acid. Kakadumycin A has wide-spectrum antibiotic activity similar to that of munumbicin D, especially against gram-positive bacteria, and it generally displays better bioactivity than echinomycin. Both echinomycin and kakadumycin A have impressive activity against *P. falciparum*, with 50% lethal doses in the range of 7 to 10 ng/ml (Castillo *et al.,* 2003). Two active ingredients identified as (i) 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and (ii) 5,7-dimethoxy-4-phenylcoumarin from endophytic *Streptomyces aureofaciens* CMUAc130 isolated from root tissue of *Zingiber officinale* Rosc. (Zingiberaceae) showed antifungal activity against *Colletotrichum musae* and *Fusarium oxysporum*. This is the first report of these compounds from microorganisms as active ingredients for the control of phytopathogenic fungi (Taechowisan *et al.,* 2005).

Antiviral compounds from fungal endophytes

Another fascinating use of antibiotic products from endophytic fungi is the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors,

cytonic acids A and B, have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. Their structures as *p*-tridepside isomers were elucidated by mass spectrometry and NMR methods (Guo *et al.*, 2000). It is apparent that the potential for the discovery of compounds, from endophytes, having antiviral activity is in its infancy. The fact, however, that some compounds have been found is promising. The main limitation in compound discovery is probably related to the absence of appropriate antiviral screening systems in most compound discovery programs.

Volatile antibiotics from endophytes

Muscodor albus is a newly described endophytic fungus obtained from small *limbs* of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong *et al.*, 2001). This xylariaceaous (non-spore-producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds (Strobel *et al.*, 2001). The majority of these compounds have been identified by gas chromatography-mass spectrometry, synthesized or acquired, and then ultimately made into an artificial mixture. This mixture mimicked the antibiotic effects of the volatile compounds produced by the fungus. It was also used to gain positive identification of the ingredients of the fungal volatile compounds (Strobel *et al.*, 2001). The ecological implications and potential practical benefits of the "mycofumigation" effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the United States. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. In fact, this organism is already on the market for the decontamination of human wastes. *Muscodor roseus* obtained from tree species growing in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus* (Worapong *et al.*, 2002). In addition, for the first time, a nonmuscodor species, a *Gliocladium* sp., was discovered to be a volatile antibiotic producer. The volatile components of this organism are totally different from those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8] annulene, formerly used as a rocket fuel and discovered for the first time as a natural product in an endophytic fungus

(Stinson *et al.*, 2003). The bioactivity of the volatile compounds of *Gliocladium* sp. is not as good or comprehensive as those of the *Muscodor* spp. (Stinson *et al.*, 2003).

Products from endophytes as antioxidants

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage of Papua New Guinea (Harper *et al.*, 2003 b, Strobel *et al.*, 2002b). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavonoids (Fig. 8).

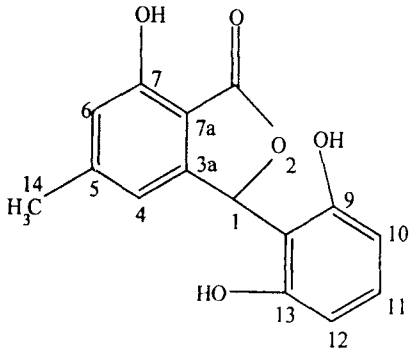

Fig. 8: Isopestacin, an antioxidant produced by an endophytic *Pestalotiopsis microspora* strain isolated from *T. morobensis* growing on the north coast of Papua New Guinea

Pestacin was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Harper *et al.*, 2003 b) (Fig. 9). Proposed antioxidant activity of pestacin arose primarily via cleavage of an unusually reactive C-H bond and to a lesser extent, though O-H abstraction (Strobel, 2002a). The antioxidant activity of pestacin is at least 1 order of magnitude greater than that of trolox, a vitamin E derivative (Harper *et al.*, 2003 b).

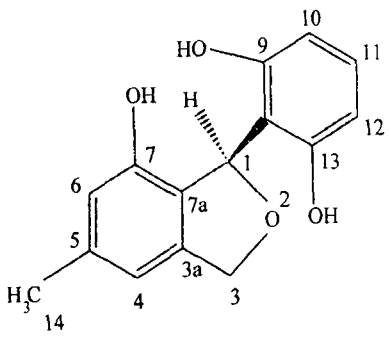

Fig. 9: Pestacin is also produced by the *Pestalotiopsis microspora* fungus and it, too, is an antioxidant

Products of endophytes with insecticidal activities

Bioinsecticides are only a small part of the insecticide field, but their market is increasing (Demain, 2000). Several endophytes are known to have anti-insect properties. Nodulisporic acids, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels. The first nodulisporic compounds were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for more *Nodulisporium* spp. or other producers of more-potent nodulisporic acid analogues (Demain, 2000). Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2- (1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2- (1'-oxo-5'-methyl-4'-hexenyl) benzofuran, both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm (Findlay *et al.,* 1997). Another endophytic fungus, *Muscodor vitigenus*, isolated from a liana (*Paullina paullinioides*), yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellant. *M. vitigenus* shows promising preliminary results as an insect deterrent and has exhibited potent insect repellency against the wheat stem sawfly (*Cephus cinctus*) (Daisy *et al.,* 2002a, Daisy *et al.,* 2002b). As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research continues for the discovery of powerful, selective and safe alternatives.

Antidiabetic agents from endophytic fungi

A nonpeptidal fungal metabolite (L-783, 281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang *et al.*, 1999). This compound acts as insulin mimetic and, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783, 281 to two mouse models of diabetes resulted in significant lowering of blood glucose levels. These results may lead to new therapies for diabetes (Zhang *et al.*, 1999).

Immunosuppressive compounds from endophytes

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future, they could be used to treat autoimmune diseases such a rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinol A and B (Lee *et al.*, 1995 b) (Fig. 10). Subglutinol A and B are equipotent in the mixed lymphocyte reaction assay and thymocyte proliferation assay, with a 50% inhibitory concentration of 0.1 μM. In the same assay systems, the famed immunosuppressant drug cyclosporine is roughly as potent in the mixed lymphocyte reaction assay and 10^4 more potent in the thymocyte proliferation assay. Still, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail (Lee *et al.*, 1995 b).

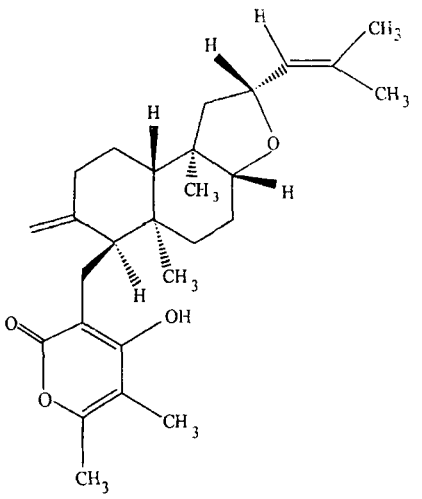

Fig. 10: Subglutinol A, an immunosuppressant, is produced by an endophytic *F. subglutinans* strain

The Microbiology Department at Sandoz Ltd. developed a computer-aided evaluation program to screen and evaluate fungi for bioactivity. This approach resulted in the discovery of the fungus *Tolypocladium inflatum*, from which cyclosporine, a hugely beneficial immunosuppressant, was isolated (Borel and Kis, 1991). *Pestalotiopsis* species have been reported to secrete immunosuppressive compounds in culture broth. *Pestalotiopsis* sp., isolated as an endophytic fungus from *T. brevifolia* (Yew tree), produces immunosuppressive pestalotiopsins A and B in liquid culture (Pulici *et al.*, 1996a). *Pestalotia* sp. (Strain AB 1942R-114), isolated from a partridge pea plant (Cassia fistula), produces immunosuppressive Cytochalasin U (Burres *et al.*, 1992). *Pestalotiopsis leucothes* HKUCC 10197, an endophytic fungus from *T. wilfordii* and shown it has tremendous immunomodulatory effects especially suppression of various activities of peripheral blood mononuclear cells (PBMNC) (Kumar *et al.*, 2003). Bioassay guided purification of the crude extracts of *P. leucothes* revealed 3 compounds that accounted for its activity (Kumar *et al.*, 2005).

Hydrolyases from endophytes

Like other microorganisms invading plant tissues, endophytes produce extracellular hydrolyases as a resistance mechanism to overcome attack by the host against pathogenic invasion and/or to get nutrition from the host. Such enzymes including

pectinases, esterases, cellulases and lipases, (Petrini *et al.,* 1992) proteinase, (Reddy *et al.,* 1996) α-1, 4-glucan lyase (Nielsen *et al.,* 2000) and phosphatases (Maccheroni and Azevedo, 1998) have been documented with different endophytes. Enzymatic activities closely related to the host-specificity of the endophytes were demonstrated (Petrini *et al.,* 1992). The action of these enzymes gives rise to the possibility that the 'genetic recombination' of the endophyte with the host may occur in evolutionary time. In addition to above-mentioned enzymes endophytes also produces laccases and Xylanases. Xylan is one of the most abundant polysaccharide in the plant cell wall. However, very few microorganisms living in plants, e.g., the ericoid mycorrhizal fungus *Hymenoscyphus ericae* producing p-1, cendoxylanase (Burke and Cairney, 1997) were reported to be xylanase producers even if one of the major components of the plant cell wall is xylan. Manabu *et al.,* (2002) isolated one hundred and sixty-nine endophytic fungi and 81 endophytic bacteria from 14 plants in total. Among them, 155 fungi (91.7%) and 52 bacteria (64%) were found to produce xylanase.

F. proliferatum NRRL 31071 strain produced laccases during infection of wheat seedlings (Kwon and Anderson, 2001). Two isolates of *Fusarium proliferatum* MUCL 31970, *F. proliferatum* NRRL 31071 from different global locations and habitats mineralized several natural and synthetic lignins. Mineralization correlated with the detection of extracellular laccase and aryl alcohol oxidase activities (Anderson *et al.,* 2005).

Anticancer agents from endophytic fungal products

Cancer is a growing public problem whose estimated worldwide new incidence is about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. It is a disease characterized by unregulated proliferation of cells. The search for natural products as potential anticancer agents dates back, at least, to the Ebers papyrus in 1550 BC, but the scientific period of this search is much more recent, beginning with the investigations by Hartwell and co-workers in late 1960s on the application of podophyllotoxin and its derivatives as anticancer agents. A large number of plants, marine and microbial sources have been tested as leads, and many compounds have survived the potential leads.

Several potential lead molecules such as camptothecin, vincristine, vinblastine, taxol, podophyllotoxin, combretastatins, etc. have been isolated from plants and many of them have been modified to yield better analogues for activity, toxicity or solubility. Several successful molecules like topotecan, irinotecan, taxotere, etoposide, teniposide, etc. also have emerged as drugs upon modification of these natural leads and many more are yet to come.

Paclitaxel and some of its derivatives represent the first major group of anticancer agents that is produced by endophytes (Fig. 11).

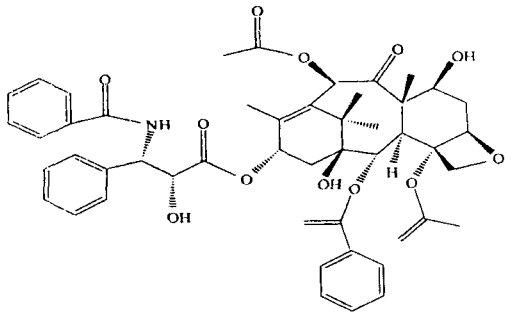

Fig.11: Paclitaxel, the world's first billion-dollar anticancer drug is produced by many endophytic fungi. It, too, possesses outstanding antioomycete activity

Paclitaxel, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species (Suffness, 1995). The mode of action of paclitaxel is to preclude tubulin molecules from depolymerizing during the processes of cell division (Schiff and Horowitz, 1980). This compound is the world's first billion-dollar anticancer drug. It is used to treat a number of other human tissue-proliferating diseases as well. The presence of paclitaxel in yew species prompted the study of their endophytes. By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel paclitaxel-producing endophytic fungus, *T. andreanae*, was discovered in *T. brevifolia* (Strobel *et al.*, 1993). In electrospray mass spectroscopy, paclitaxel usually gives two peaks, one at a mass of 854, which is $(M + H^+)$, and the other at 876, which is $(M + Na^+)$, and fungal paclitaxel had a mass spectrum identical to that of authentic paclitaxel (Stierle *et al.*, 1993). Then, ^{14}C labeling studies irrefutably showed the presence of fungus-derived paclitaxel in the culture medium (Stierle *et al.*, 1993.). Some of the most commonly found endophytes of the world's yews are

Pestalotiopsis spp. (Strobel *et al.,* 1996). One of the most commonly isolated endophytic species is *P. microspore* (Strobel, 2002a). An examination of the endophytes of *Taxus wallichiana* yielded *Pestalotiopsis microspora*, and a preliminary monoclonal antibody test indicated that it might produce paclitaxel (Strobel *et al.,* 1996). Labeled (^{14}C) paclitaxel was produced by this organism from several ^{14}C precursors that had been administered to it (Strobel *et al.,* 1996). Furthermore, several other *Pestalotiopsis microspora* isolates were obtained from bald cypress in South Carolina and also shown to produce paclitaxel (Li *et al.,* 1996). This was the first indication that endophytes residing in plants other than *Taxus* spp. were producing paclitaxel. From the extremely rare, and previously thought to be extinct, Wollemi pine (*Wollemia nobilis*), *Pestalotiopsis guepini* was isolated, which was shown to produce paclitaxel (Strobel *et al.,* 1997). Also, quite surprisingly, a rubiaceous plant, *Maguireothamnus speciosus*, yielded a novel fungus, *Seimatoantlerium tepuiense*, that produces paclitaxel. Furthermore, fungal paclitaxel production has also been noted in a *Periconia* sp. (Li *et al.,* 1998) and in *Seimatoantlerium nepalense*, another novel endophytic fungal species (Bashyal *et al.,* 1999). Simply, it appears that the distribution of those fungi making paclitaxel is worldwide and not confined to endophytes of yews. The ecological and physiological explanation for the wide distribution of fungi that make paclitaxel seems to be related to the fact that paclitaxel is a fungicide and the organisms with the most sensitivity to it are plant pathogens such as *Pythium* spp. and *Phytophthora* spp. (Young *et al.,* 1992). These pythiaceous organisms are some of the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants. In fact, their sensitivity to paclitaxel is based on their interaction with tubulin in a manner identical to that in rapidly dividing human cancer cells (Schiff and Horowitz, 1980). As time has passed, other investigators have also made observations on paclitaxel production by endophytes, including the discovery of paclitaxel production by a *Tubercularia* sp. isolated from southern Chinese yew (*Taxus mairei*) in the Fujian province of southeastern China (Wang *et al.,* 2000). At least three endophytes of *T. wallichiana* produce paclitaxel, including *Sporormia minima* and a *Trichothecium* sp. (Shrestha *et al.,* 2001). By the use of high-performance liquid chromatography and electrospray mass

spectroscopy, paclitaxel has been discovered in *Corylus avellana* cv. Gasaway (filbert) (Hoffman *et al.*, 1998). Several fungal endophytes of filbert produce paclitaxel in culture (Hoffman *et al.*, 1998). It is important to note, however, that paclitaxel production by all endophytes in culture is in the range of submicrograms to micrograms per litre. Also, commonly, endophytic fungi will attenuate paclitaxel production in culture. It is possible, however, to recover paclitaxel production in attenuated cultures if certain activator compounds are added to the medium (Li *et al.*, 1998). Torreyanic acid, a selectively cytotoxic quinone dimer (anticancer agent), was isolated from a *Pestalotiopsis microspora* strain. This strain was originally obtained as an endophyte associated with the endangered tree *T. taxifolia* (*Florida torreya*) as mentioned above (Lee *et al.*, 1996). Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5 to 10 times more potency in those lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis. Recently, a complete synthesis of torreyanic acid has been successfully completed using the application of a biomimetic oxidation-dimerization cascade (Li *et al.*, 2003).

The alkaloids are also commonly found in endophytic fungi. Such fungal genera as *Xylaria*, *Phoma*, *Hypoxylon*, and *Chalara sp.* are representative producers of a relatively large group of substances known as the cytochalasins, of which over 20 are now known (Wagenaar *et al.*, 2000). Many of these compounds possess antitumor and antibiotic activities, but because of their cellular toxicity they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from a *Rhinocladiella* sp. as an endophyte on *Tripterygium wilfordii*. These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins (Wagenaar *et al.*,2000).

The extract of a *Curvularia* sp. an endophytic fungus isolated from *Ocotea corymbosa* (Meissn) Mez. showed moderate antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides*, triggering further studies. The crude EtOAc extract afforded two new benzopyran derivatives and two known compounds and which were tested in cell proliferation and antifungal assays. Compound (2'S)-2-(propan-2'-ol)-5-hydroxy-benzopyran-4-one caused a potent proliferative stimulus to two mammalian cell lines and exhibited a weak antifungal

activity (Teles *et al.*, 2005). Recently fractionation of the extract of *Aspergillus niger* IFB-E003, an endophyte in *Cyndon dactylon*, gave four known compounds naphtho-γ-pyrones rubrofusarin B, fonsecinone A, asperpyrone B and aurasperone A, which were further investigated biologically. Rubrofusarin B was shown to be cytotoxic to the colon cancer cell line SW1116 (IC_{50}: 4.5 μg ml^{-1}), and aurasperone A inhibitory on XO (xanthine oxidase) (IC_{50}: 10.9 μgmol^{-1}) (Song *et al.*, 2004). Recently, podophyllotoxin-producing endophytes have been discovered from various *Podophyllum* plant species (Porter and Eyberger, 2004). Also RRL, Jammu reported the production of lignans by a novel fungal endophyte (*Trametes hirsuta),* which are biologically active, and exhibit potent antioxidant, anticancer and radioprotective properties (Puri *et al.*, 2005b). But there was no published report of production of CPT from endophytic fungi. Here in this study an endophytic fungus *Entrophospora infrequens* associated with *Nothapodytes foetida*, a medicinal plant native to Indian Western Ghats was found to produce CPT and its derivatives.

Camptothecin

Camptothecin (Wall *et al.*, 1966), a naturally occurring alkaloid, is a potent antitumor compound (Gallo *et al.*, 1971). It is an inhibitor of DNA topoisomerise I (Jaxel *et al.*, 1989). The compound was originally isolated (Wall *et al.*, 1966) from the Chinese plant, *Camptotheca acuminata* in very low yield. Indian *Nothopodytes foetida* (formerly, *Mappia foetida*) has been discovered (Govindachari *et al.*, 1972b) as a rich source of the compound. The Indian species also afforded (Govindachari *et al.*, 1972b; Srinivas and Das, 2003) three other antitumor alkaloids, 9-methoxycamptothecin, 20-*O*-acetylcamptothecin and 20-*O*-acetyl-9-methoxy-camptothecin. The lactone moiety in the E-ring of camptothecin is the most important structural feature with respect to their antitumor property (Wall and Wani, 1977). The decarboxylated E-ring analogues of camptothecin and 9-methoxycamptothecin, known as mappicine ketone and 9-mehtoxymappicine ketone respectively are also minor constituents of *Nothopodytes foetida.* (Das *et al.*, 1998a; Das *et al.*, 1998b; Dasand Madhusudhan, 1999). These two compounds have recently been identified as promising antiviral compounds. (Pendrak *et al.*,

1995). Mappicine ketone and 9-methoxymappicine ketone are the oxidized forms of the natural antitumor alkaloids, (*S*)-mappicine and (*S*)-9-methoxymappicine respectively, which are also present in *Nothapodytes foetida* (Das *et al.*, 1998a; Das *et al.*, 1998b; Das and Madhusudhan, 1999). Camptothecin molecule became so important that during 1966–2004 over 3000 research papers appeared on it. Presently, the first generation analogues of CPT, hycamtin (topotecan) and camptosar (irinotecan, CPT-11), marketed by Glaxo-Smith Kline (GSK) and Pfizer, respectively, are used for the treatment of ovarian and colon cancers (Saltz *et al.*, 2000; Gore *et al.*, 2001).

The specific physical and chemical properties of camptothecin are shown in Table 2.

Distribution of camptothecin and its metabolites

Since secondary metabolites are often similar within members of a clade, their occurrence or absence might be taken as an indication of common descent and thus relatedness. Chemotaxonomists have compared the known distribution patterns of CPT with the phylogenetic system of classification of the angiosperms, finding surprising results (Wink, 2003). Quite often, even allelo chemicals of high structural specificity and complexity occur simultaneously in unrelated families of the plant kingdom. The multifunctional monoterpenoid indole alkaloid (TIA) CPT is an illustrative example of this kind of metabolites. As shown in Fig. 12, CPT has been isolated from samples of the following unrelated orders and families of angiosperms: Order Celastrales: *Nothapodytes foetida* (Aiyama *et al.*, 1998), *Pyrenacantha klaineana* (Zhou *et al.*, 2000), and *Merrilliodendron megacarpum* (Arisawa *et al.*, 1981; Icacinaceae): Order Cornales: *C. acuminata* (Wall *et. al.*, 1966), *C. lowreyana*, and *C. yunnanensis* (Li *et. al.*, 2002; Nyssaceae); Order Gentianales: *Ophiorrhiza mungos* (Tafur *et. al.*, 1976), *O. pumila*, *O. filistipula* (Saito *et al.*, 2001; Rubiaceae), *Ervatamia heyneana* (Gunasekera *et al.*, 1979; Apocynaceae), and *Mostuea brunonis* (Dai *et al.*, 1999; Gelsemiaceae).

Table 2: Physical and chemical properties of camptothecin

Properties	Specification
Chemical name	4-Ethyl-4-hydroxy-1H-Pyrano-[3;4:6,7]indolizinol [1,2-b] quinoline-3, 14 (4H, 12H)-dione.
Chemical family	Alkaloid
Molecular formula	$C_{20}H_{16}N_2O_4$
Molecular weight	348.34
Physical state	Pale yellow needles
Melting point	275-277°C
Solubility	Chloroform: methanol (8:2 v/v)

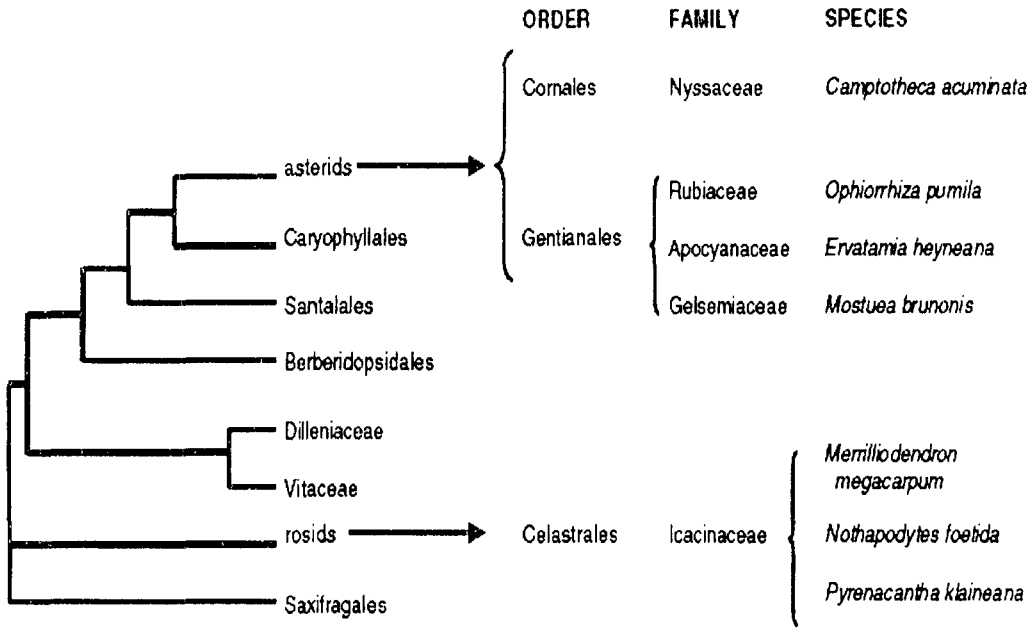

Fig. 12:CPT has been identified in plant species belonging to unrelated order and families of angiosperms (based on reviews of Gentianales and angiosperm phylogeny, Backlund *et al.*, (2000) and Hilu *et al.*, (2003), respectively)

Biological activity

CPT is a potent cytotoxic agent. It shows anticancer activity mainly for solid tumours. It inhibits DNA topoisomerase I (Redinbo *et al.,* 1998, Staker *et al.,* 2002). It shows anticancer activity mainly against colon and pancreatic cancer cells. But its analogues showed anticancer activity in breast, liver, prostate, etc.

Future prospects

CPT and its analogues exhibit a broad spectrum of antitumour activity and represent a very promising class of agents. The discovery of topoisomerases as new targets for cancer chemotherapy and the mechanism of action of camptothecin put camptothecin back on the frontlines of anticancer drug development. (Potmesil and Pinedo, 1995b). Two of the successful drugs, topotecan and CPT-11, have achieved nearly US $750 million in annual sales. Camptothecin will continue to remain a target for new synthetic methods, which are certainly expected considering the fast development of modern organic synthesis. Continued studies on the camptothecin–DNA–topoisomerase I interaction in addition to its detailed mechanism of action may suggest new directions in the synthesis of new camptothecins.

Ergosterol

Ergosterol is the primary sterol in the cell membranes of filamentous fungi and is either absent or a minor component in most higher plants. It is also present in membranes in the yeast cell wall and mitochondria (Axelsson *et al.,* 1995; Matile *et al.,* 1969). Ergosterol is a constituent of membranes in mycelia, spores and vegetative cells (Newell, 1992). Ergosterol content has been widely used as an estimate of fungal biomass in various environments, e.g., in soil and aquatic systems, because a strong correlation has been found between ergosterol content and fungal dry mass (Matchan *et al.,* 1985; Newell, 1992; Newell, 1994; Schnurer, 1993; Suberkropp *et al.,* 1993). However, the amount of ergosterol in fungal tissue is not constant. There are interactions between the amount of ergosterol and fungal species, age of the culture, developmental stage (growth phase, hyphal formation and sporulation), and growth conditions (growth media, pH, and temperature),

although no clear trend for the ergosterol content in any of these factors has yet been detected (Gessner *et al.*, 1993; Newell, 1994; Schnurer, 1993).

Ergosterol has also been suggested for use in quantifying fungal growth in solid substrates because of a good correlation between the ergosterol content and hyphal length (Schnurer, 1993). Ergosterol measurements were proposed as a new method for determination of total fungal biomass in investigations of indoor environments (Flannigan, 1997; Miller *et al.*, 1997). The specific physical and chemical properties of ergosterol are shown in Table 3.

5-Hydroxymethyl-2-furfuraldehyde

On heating carbohydrate solutions, the presence of many products has been demonstrated (Popoff and Theander, 1976; Hodge and Osman, 1976; Olsson *et al.*, 1977; Olsson *et al.*, 1978; Nilsson-Thorel *et al.*, 1993). Nevertheless, the main breakdown product seems to be 5-hydroxymethyl-2-furfuraldehyde (5-HMF), which justifies its selection for quality control purposes (Lee and Nagy, 1988); its concentrations are helpful and frequently used as an indicator of time of storage and/or to measure the degree of thermal abuse in carbohydrate foodstuffs. Several authors have successfully determined this compound in several products of caramel (Alfonso *et al.*, 1980), tomato paste (Allen and Chin, 1980; Porretta and Sandei, 1991), fruit juices (Mijares *et al.*, 1986; Fuleki and Pelayo, 1993), wines (Williams *et al.*, 1983), plant extracts (Kiridena *et al.*, 1994), pharmaceutical syrups and infusion fluids (Cook *et al.*, 1989), honey (Salinas *et al.*, 1991; Vinas *et al.*, 1992), milk (Morales *et al.*, 1992), khoa (Sahaia *et al.*, 1992), brandies (Mir *et al.*, 1992), baby cereals (Guerra-Hernandez *et al.*, 1992) and coffee (Chambel *et al.*, 1997). However, the well-known combinations of 5-HMF with some food components, and the chemical instability of the compound, may diminish its usefulness as a chemical marker of quality of food, particularly when strong thermal or prolonged heating treatments of food are employed.

The specific physical and chemical properties of 5-HMF are shown in Table 4 .

Table 3: Physical and chemical properties of ergosterol

Properties	Specification
Chemical name	Ergosta-5,7,22-trien-3-ol
Chemical family	Steroids
Molecular formula	$C_{28}H_{44}O$
Molecular weight	396.63
Physical state	Amorphous
Melting point	166-183°C
Solubility	Chloroform, Alcohol, ether

Table 4: Physical and chemical properties of 5-HMF

Properties	Specification
Chemical name	5-(Hydroxymethyl)-2-furfuraldehyde
Chemical family	Furan
Molecular formula	$C_6H_6O_3$
Molecular weight	126.11
Physical state	Solid
Melting point	31.5
Solubility	Ether, benzene, chloroform less sol in carbon Tetrachloride

Endophytes and ethnobotany

A comprehensive study on the endophytes of any individual rainforest higher plant species has not been done, much less a study on any individual plant in its entirety, from its complete root system to its stems, petioles, leaves and flowers. The prospects of finding endophytes (fungi and bacteria) that are specific to any given higher plant or even occurring only in a local region in a forest seem great, given the paucity of work in this area. Other areas of the world holding fascinating plants and associated endophytes are Northwestern Himalayas, Western Ghats, the tepuis of Venezuela, the rainforests of Central America, and the monsoonal areas of Australia. In addition, the golden triangle of Thailand, and the highlands and coastal areas of Papua New Guinea, the entire country of Madagascar, and the upper Amazon regions are other areas with great biodiversity (Mittermeier *et al.*, 1999). In each of these areas, novel endophytic fungal taxa have been discovered as well as a series of new bioactive compounds. Again, each of these areas of the world has abundant rainfall, wide plant species diversity, and many endemic plants. The search for novel endophytes and their associated secondary metabolites products should also be directed towards plants that commonly serve native populations for medicinal purposes. It is conceivable that these plants have microbes that mimic the chemistry of their respective host plants and make the same bioactive natural product(s) or derivatives that are more bioactive than those of their respective host. This is exemplified with the case of taxol from yews and also taxol being produced by a series of endophytes from yews as well as other plant sources. Thus, if a microbial source for a medicinally important substance can be found, then its supply is better guaranteed than if its sole source is from one or more obscure, rare, or difficult to cultivate higher plants. Overall, the prospects for scientific discovery in this emerging area seem promising. Equipped with novel bioassay systems and modern chemical separation science, many new products for medicine are likely to result.

In summary, endophytes have provided many exciting scientific advancements and practical benefits. However, there is much more to look forward to in future.

In the light of above, it is, therefore, envisaged to explore the Himalayan as well as Jammu region *Nothapodytes foetida* plants for the isolation of endophytic fungi, which may eventually be utilized to produce bioactive molecules / metabolites of therapeutic value particularly comptothecin.

CPT and its analogues exhibit a broad spectrum of antitumour activity and represent a very promising class of anti-cancer agents. Although, CPT is a promising anti-tumour agent, it is, however, available only in low concentration in tree roots, which demands uprooting of rare and old trees. Thus, if a microbial source for such a medicinally important substance (CPT) can be found, then its supply is bettrer guaranteed. But there was no published report of production of CPT from endophytic fungi. In the present study, an endophytic fungus identified as *Entrophospora infrequens* associated with *N. foetida* of Jammu region was found to produce CPT. *N. foetida* plants were for the first time explored for endophytic microbes.

The ultimate aim of present study was to explore these medicinally important plants for the isolation of endophytic fungi, which may be useful for biotransformation and *de novo* synthesis of novel bioactive principles.

AIMS AND OBJECTIVES

For the proposed investigation, a strain of endophytic fungus designated as RJMEF001 with a capacity to produce CPT molecule has been selected. The strain has been investigated for the production, purification and characterization of CPT. Besides; the other minor chemical constituents of this fungal endophyte have also been isolated and characterized. The following steps were followed to achieve the aims and objectives of the study:

- Isolation of endophytic fungi from *N. foetida* plants growing at RRL campus Jammu and from explant samples collected from Mahableshwar forest.
- Purification and microscopic studies of isolated strains.
- Scanning electron microscopic studies of endophytic fungal spores.
- Identification of the fungus by 16S ribosomal gene typing
- Screening of these isolated microbial strains for the production of CPT.
- Isolation of CPT and 9-methoxycamptothecin from host plant by semi-preparation method.
- Shake flask studies of RJMEF001 isolate for production of CPT.
- Optimization of fermentation medium and other parameters for optimum fungal growth followed by production of Camptothecin in shake flasks.
- Study of growth and production kinetics of RJMEF001 isolate in best-suited medium in shake flasks.
- Solid-state fermentation studies of RJMEF001 isolate.
- Bioreactor studies for the production of CPT by endophytic fungus (RJMEF001).
- Effect of precursor molecules on the yield of fungal CPT.
- Isolation, purification and characterization of the fungal CPT.
- Quantitation of CPT in fungal extracts by LC-MS/MS.
- Isolation of other chemical constituents produced by RJMEF001 isolate.
- Screening of mycelial extracts for *in vitro* cytotoxicity against human cancer cell lines.

CHAPTER 3

BIOLOGICAL STUDIES ON *N. FOETIDA* ASSOCIATED ENDOPHYTIC FUNGUS

Materials and Methods

The chemicals/reagents used in the present study and their sources are listed below

Chemicals	Source
Acetone	Qualigens Fine Chemicals, India
Acetonitrile	Ranbaxy Fine Chemicals, India
Agar-Agar	Hi-Media, India
Agarose	Hi-Media, India
Ammonium oxalate	Ranbaxy Fine Chemicals, India
Beef extract	Hi-Media, India
Benzene	Qualigens Fine Chemicals, India
Calcium sulphate	Ranbaxy Fine Chemicals, India
Camptothecin	Sigma chemical company, India
Chloroform	Ranbaxy Fine Chemicals, India
Citral	Sigma-Aldrich Corp., India
Copper sulphate	Qualigens Fine Chemicals, India
Dextrose	S.D. Fine Chemicals, India
Difco Yeast	S.D. Fine Chemicals, India
Dimethyl sulphoxide	Ranbaxy Fine Chemicals, India
Ethanol	E. Merck, Germany
Ethylene diamine tetra acetate	Hi-Media, India
Ferric sulphate	Ranbaxy Fine Chemicals, India
Ferrous sulphate	Ranbaxy Fine Chemicals, India
Fetal calf serum (FCS)	Sigma Chemicals Co., USA
5-Flurouracil	Sigma Chemicals Co., USA
Gentamycin	Sigma Chemicals Co., USA
Geraniol	Sigma-Aldrich Corp., India
Glacial acetic acid	Ranbaxy Fine Chemicals, India
Glucose	Ranbaxy Fine Chemicals, India

Glutaraldehyde	E. Merck, Germany
Glycerol	Hi-Media, India
Hexane	Qualigens Fine Chemicals, India
Human cancer cell lines	National center for cell Science, Pune, India
Human cancer cell lines	National center for cell Science, Fredrick, USA
Isopropyl alcohol	Sisco Research Laboratories, India
Lactophenol cotton blue	Hi-Media, India
Leucine	Hi-Media, India
Magnesium sulphate	Qualigens Fine Chemicals, India
Malt extract	Hi-Media, India
Maltose	Ranbaxy Fine Chemicals, India
Methanol	Ranbaxy Fine Chemicals, India
Mevalonic acid	Hi-Media, India
Minimum essential medium (MEM)	Sigma Chemicals Co., USA
Mitomycin C	Sigma Chemicals Co., USA
Osmium tetroxide	E. Merck, Germany
Paclitaxel	Sigma Chemicals Co., USA
Penicillin	Sigma Chemicals Co., USA
Peptone	Hi-Media, India
Phosphate buffer saline (PBS)	Sigma Chemicals Co., USA
Potassium chloride	Ranbaxy Fine Chemicals, India
Potassium dihydrogen orthophosphate	Qualigens Fine Chemicals India
Potassium nitrate	Ranbaxy Fine Chemicals, India
Potassium phosphate dibasic	Ranbaxy Fine Chemicals, India
Rnase	Bio Basic, Canada
Roswell Park Memorial Institute medium (RPMI)	Sigma Chemicals Co., USA
Silica gel	Qualigens Fine Chemicals, India

Silicon	Hi-Media, India
Sodium bicarbonate	Hi-Media Laboratories, India
Sodium chloride	Ranbaxy Fine Chemicals, India
Sodium sulphate	Qualigens Fine Chemicals, India
Starch	Qualigens Fine Chemicals, India
Streptomycin	Sigma chemical company, USA
Sucrose	Ranbaxy Fine Chemicals, India
Sulphorhadamine	Hi-Media, India
Sulphorhodamine Blue (SRB)	Sigma Chemicals Co., USA
Trichloroacetic acid	Ranbaxy Fine Chemicals, India
Tris acetate	Hi-Media, India
Tris base	Sigma chemical company, USA
Trypsin	Sigma Chemicals Co., USA
Tryptophan	Hi-Media, India
Tween 80	Hi-Media, India
Urea	S.D. Fine Chemicals, India
Water	Ranbaxy Fine Chemicals, India
Yeast extract	Hi-Media, India

(All other chemicals/reagents used were also of analytical grade, water was glass double distilled, and solvent were of HPLC grade).

Miscellaneous Items

Millipore filters (pore size 0.25 and 0.45μm) were purchased from Millipore Pvt. Ltd. Banglore, India. Whatman filter papers (No.1) were from Whatman International Ltd, Maidstone, England. Parafilm 'M' was obtained from America Can Company, CT, USA. pH indicator papers were obtained from Qualigens Fine Chemicals, Mumbai, India. Aluminum foil was obtained from S.R Foil Ltd. New Delhi, India. Petriplates and Eppendorf tubes were obtained from Tarson products Pvt. Ltd. New Delhi, India. TLC plates were purchased from Merk, Darmstadt, Germany.

Media used

The compositions of all media (gl^{-1}) used in this study are as follows:

(18-20g of agar per litre was added for the preparation of solid media)

Czapek broth (M_1, pH 7.0)

Ammonium oxalate 3.0g; Potassium hydrogen phosphate 1.0g; Magnesium sulphate 0.5g; Potassium chloride 0.5g; Ferric sulphate 0.05; Sucrose 30g

Czapek broth (M_2, pH 7.0)

Dextrose 30g; Ammonium oxalate 3.0g; Potassium hydrogen phosphate 1.0g; Magnesium sulphate 0.5g; Potassium chloride 0.5g; Ferric sulphate 0.05g

Malt extract broth (M_3, pH 7.0)

Malt extract 20g; Glucose 20g; Peptone 1.0g

Yeast Beef extract broth (M_4, pH 7.2)

Yeast extract 1.0g; Beef extract 1.08 g; Peptone 2.0g; Ferrous sulphate 0.001g; Glucose 10g

Yeast malt extract broth (M_5, pH 7.0)

Malt extract 10g; Yeast extract 4g; Magnesium sulphate 0.5g; Potassium hydrogen phosphate 0.5g

Molasses broth (M_6, pH 7.2)

Molasses 7.5g; Sodium chloride 10g; Calcium sulphate 0.250g; Corn steep liquor 20g; Ferrous sulphate 0.160g; Copper sulphate 0.100g; Magnesium sulphate 0.500g; Potassium hydrogen phosphate 0.6g

Goose & Tschessch broth (M_7, pH 7.0)

Peptone 2; Glucose 10g; Magnesium sulphate 0.5g; Potassium hydrogen phosphate 0.5g

Malt extract broth (M_8, pH 5.4)

Malt extract 30g; Peptone 5.0g

Potato Dextrose broth (M_9, pH 6.0)

Diced Potato extract 300g; Dextrose 20g

Glucose yeast broth (M_{10}, pH 7.0)

Peptone 5g; Glucose 20g; Difco yeast 2.0g

Leonine broth (M_{11}, pH 7.2)

Peptone 0.625g; Maltose 6.25g; Malt extract 6.25 g; Potassium hydrogen phosphate 1.25g; Magnesium sulphate 0.625 g

Bianchi broth (M_{12}, pH 7.0)

Potassium hydrogen phosphate 1.0g; Potassium nitrate 1.0g; Magnesium sulphate 0.55g; Potassium chloride 0.5g; Starch 0.2 g; Glucose 0.2g; Sucrose 0.2g

Czapek broth (M_{13}, pH 7.0)

Dextrose 30g; Urea 3.0g; Potassium hydrogen phosphate 1.0g; Magnesium sulphate 0.5g; Potassium chloride 0.5g; Ferric sulphate 0.05g

Czapek broth (M_{14}, pH)

Sucrose 30g; Urea 3.0g; Potassium hydrogen phosphate 1.0g; Magnesium sulphate 0.5g; Potassium chloride 0.5g; Ferric sulphate 0.05g

Malt salt broth (M_{15}, pH 7.0)

Malt extracts 100g; Sodium chloride 100g

Malt sucrose broth (M_{16}, pH 7.0)

Malt extract 20g; Sucrose 200g

Yeast malt extract dextrose broth (M_{17}, pH 7.0)

Malt extract 10g; Dextrose 4g; Yeast extract 4g

Sabouraud broth (M_{18}, pH 5.6)

Dextrose 40g; Peptone 10g

Sabouraud broth (M_{19}, pH 5.6)

Dextrose 40g; Peptone 10g; Magnesium sulphate 0.5g; Potassium dihydrogen orthophosphate 1.0g

Water agar

15 g agar in distilled water

Buffers and solutions

Normal saline solution (NSS)

8.68 g Sodium chloride in 1litre distilled water (pH 7.0$\pm$ 0.2)

TAE buffer

100 ml Concentrated stock solution (50 X)

Tris base 242 g; Glacial acetic acid 57.1 ml; EDTA 186 g (pH 8.0)

1.0 M Tris. HCl (pH 8.0)

121gl^{-1} Tris. pH was adjusted with dilute HCl

0.5 M EDTA (pH 8.0)

EDTA 186 gl^{-1}, pH was adjusted with 2N NaOH

TES buffer (pH 7.5)

100 mM Tris. HCl -12.1 gl^{-1}; 25mM EDTA-9.3 gl^{-1}; 2% SDS-20 gl^{-1}

TE buffer (pH 8.0)

100 mM Tris. 12.1 gl^{-1}; 10mM EDTA- 3.72 gl^{-1}

Ethanol (70%)

Ethanol 700 ml; 300ml distilled water

Ethanol (95%)

Ethanol 950 ml; 50ml distilled water

DNA Loading dye

40% (w/v) Sucrose-400 gl^{-1}; 0.25% (w/v) Bromophenol blue-2.5 gl^{-1}

Glutaraldehyde, Osmium tetroxide and washing buffer

Solution A

4.25% $NaH_2PO_4.H_2O$

Solution B

5.04% NaOH

Solution C

10% Sucrose

Solution D

41.5 ml Sol. A + 7 ml Sol. B (pH was adjusted 7.2 with sol. B)

Solution E

2% OsO_4

Solution F

25% Glutaraldehyde

Glutaraldehyde fixative (100 ml) (2.5%)

45 ml Sol. D+ 10 ml Sol. C+ 10 ml Sol. F + 35 ml DDW

(Stable for 2 months at 4°C)

Osmium tetroxide Fixative (5 ml) (~1%)

2.25 ml Sol. D+ 0.5 ml Sol. C+2.25 ml Sol. E

(Stable for 3 to 4 weeks at 4°C)

Buffer for washing (100 ml)

45 ml Sol. D+45 ml DDW+20 ml Sol. C

Different carbon sources used

Glucose monohydrate, Sucrose, Starch, Maltose

Different organic / inorganic nitrogen sources used

Peptone, Yeast extract, Beef extract, Corn steep liquor, Urea, Potassium nitrate, Ammonium oxalate

Different metabolic precursors used

Tryptophan, Tryptoamine, Leucine, Geraniol, Citral, Mevalonic acid

Antibiotic

Streptomycin solution

Stock solution of streptomycin 50 $mgml^{-1}$ in DDW sterilized by micron filtration (0.2μm filter) and stored in aliquots at -20°C. Streptomycin was used at the conc. of 50 $\mu g\ ml^{-1}$

Source of endophytes

Nothapodytes foetida (*Mappia foetida*) seeds collected from Mahableshwar forest (160 00'-180 00' N and 730 30'-750 00' E, altitude 150-600 m) and grown successfully at Regional Research Laboratory, botanical garden, Jammu in the year 1995-96 (Sharma *et al.*, 2000). The main study has been made from samples collected from RRL campus Jammu which lies in 32° 44'N and 74° 55'E at approximately 400m (Fig. 13 a) altitude above see level (ASL) and is situated in the subtropical part, has markedly periodic climate, characterized by a dry and increasingly hot season from March to June, a warm monsoon period from July to September and a dry and cold weather from October to December, with little rain during the months of January and February. Jammu experiences great extremes of temperature with average maximum temperature of ~40°C and average minimum temperature ~27°C in summer and average maximum temperature ~19°C and minimum temperature ~7°C during winter. Relative humidity is minimum during April to June and maximum from July to September. In the rainy season, there is a marked rise in humidity, attaining an average maximum of ~83% in the morning and 66% in the afternoon during August.

Collection, identification and authentication of the plant material

Fresh plant material of *Nothapodytes foetida* was collected from Botanical garden of Regional Research Laboratory, Jammu (Jammu and Kashmir, North Western Indian State) and Mahabaleshwar forest (Maharashtra, West Indian State). Voucher specimen has been deposited in RRL Herbarium Jammu (Accession no 27002-04). The plant (Fig. 13 b) naturally grows in Western Ghats, and was introduced by Dr. S. N. Sharma in the agro climatic conditions of Jammu and authenticated on the basis of botanical characteristics by taxonomist Dr. B.K. Kapahi at RRL, Jammu.

Storage and preservation

Small stem pieces were cut from the plant and placed in sealed pre-labeled plastic bags after excess moisture was removed and stored at 4°C until isolation procedures could begin.

Fig. 13:(a) District map of Jammu & Kashmir indicating outline of study area

Fig. 13:(b) *Nothapodytes foetida* growing in botanical garden of Regional Research Laboratory, Jammu

Isolation and screening of CPT producing endophytes

Nothapodytes foetida plant samples collected from Mahabaleshwar forest and RRL campus Jammu were taken for the isolation of endophytes. The fungus (RJMEF001) that was the subject of this study was one of many endophytic fungi isolated from small twigs of *N. foetida* plant growing in the agro climatic conditions of Jammu, India. The fungal endophytes were isolated using a method described by Arnold *et al.*, (2000). The explant samples were treated with 90% (v/v) ethanol, allowing the alcohol to evaporate and removing the outer bark with sterilized sharp blade. Small pieces (2.5-3.5cm) of inner bark were placed on the surface of water agar (1.5% w/v) in Petriplates (Tarsons, Kolkata, India), supplemented with antibiotic streptomycin ($50\mu g ml^{-1}$). After several days, fungi were observed growing from the stem fragments in the plates. Individual hyphal tips of the various fungi were removed from the agar plates and placed on either Potato dextrose agar (PDA) or Sabouraud agar medium, and incubated at $28 \pm 2^{o}C$ for at least 12 days. Each fungal culture was checked for purity and transferred to another plate by hyphal tip method. Similar procedure, but without surface sterilization, was used as a negative control to check for surface contaminated fungi. To establish the *de novo* production of CPT by the isolated fungi, the growing mycelia were serially transferred several times to fresh mycological agar to eliminate the possibility of the fungal hyphae carrying even minute amounts of CPT from the initial plant material. In this study, we were able to acquire at least fifty-two different endophytic colonies. Each such colony was grown in liquid Sabouraud medium (100 ml) and screened for CPT production. Mycelia of fully-grown cultures were extracted with chloroform: methanol solvent (8:2v/v) and the extracts were subjected to TLC using chloroform: methanol (9:1 v/v) mobile phase and monitored under UV light at 256 nm.

Maintenance and storage

One colony indicating the presence of CPT was designated as RJMEF001 and was routinely maintained on sabouraud agar containing glucose $40gl^{-1}$, peptone $10gl^{-1}$

and agar $20gl^{-1}$ with pH 5.6. The colony was stored in the form of its spores as well as vegetative form in 15% (v/v) glycerol at -70°C.

Geographical occurrence

The fungus was found in plant samples of *N. foetida,* originally growing in the Western Ghats and later introduced in temperate region of Jammu in India. Several fungal strains were isolated from the explants of these trees. Following the serial dilution techniques, a pure axenic culture was obtained which was later identified as *Entrophospora infrequens* by adopting standard laid down procedures as described on page 68.

Sporulation

Specific sporulation medium described by Khajuria *et al.,* (2001) was used to generate profuse sporulation. Molasses medium was prepared in Roux bottles and autoclaved at 121°C for 15 min and used to produce spores. The bottles were inoculated with spore suspension and incubated at 28 ± 2°C. The spores were harvested from solid culture after 8 days of incubation period as profuse sporulation was observed.

Harvesting of spores

The spores from Roux bottles were harvested with wetting agent Tween-80 (0.01%, aqueous solution) which dislodges the spores out of the mycelium mass. The suspension was filtered through Whatman no.1 filter paper fixed on filter assembly (Tarsons, Mumbai, India) and filtrates were pooled and spores counted after appropriate dilution in heamocytometer (Counting Chamber-Nuebaur-Germany).

Simple microscopy

Microscopic examination was made under light microscope (Olympus, USA) after staining the mycelium with Lactophenol cotton blue (Cappiccino and Sherman, 1996). Photographs were taken by using a digital camera (Camedia, C-2100 ultra zoom, Tokyo, Japan).

Scanning electron microscopy

Spores of the fungus obtained by growing it on molasses medium were fixed and processed using the modified method of Millonig (1961). The spores were taken on a clean cover slip and fixed in 2.5% (v/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 2 h. The material was post fixed in 1%(v/v) Osmium tetroxide in the same buffer for three hours and dehydrated in graded ascending acetone gradient (10-100%) and dried using carbon dioxide. The samples were then mounted on stubs, coated first with carbon in vacuum evaporator (JEOL-JEE4X) and then coated with gold in a sputter coater (Polaron). The samples were finally observed in a (JEOL-100CXII) electron microscope with ASID at 40 KV.

Identification of endophyte and authentication by using homology modeling

Isolation of Total Genomic DNA

The total genomic fungal DNA was extracted from fresh mycelium of RJMEF001 isolate with Maicas *et al.,* (2000) method with modifications. *Entrophospora infrequens* spores were inoculated in liquid sabouraud medium and incubated for 24 h at 28±2°C. Young germlings were harvested by filtration, washed with sterilized distilled water and resuspended in TES buffer (pH 7.5). Approximately 0.9 g (wet weight) fresh mycelia were disintegrated with 600-micron glass beads with motor and pestle under sterile conditions. The crushed mycelia were transferred into centrifuge tube (5 ml) and cell debris was removed by centrifugation at 10,000 rpm for 20 min. The supernatant containing total DNA was subjected to de-proteinnisation by treatment with equal volume of phenol: chloroform (1:1v/v) and the residual phenol was removed by centrifugation at 10,000 rpm for 15 min and the nucleic acids were collected by precipitation with isopropanol (300 µl). The RNA was eliminated by treatment with RNase (15µl). The DNA pellet was vacuum dried and finally resuspended in TE (100µl) buffer (pH 8.0). Sample (5µl) and dye (2µl) were thoroughly mixed before loading in agarose (0.7%w/v) gel.

Quantification of DNA

Absorbance of the purified DNA at 260 nm and 280 nm were taken at appropriate dilutions and the concentration calculated as follows:

A_{260} of 1 = 50 µg DNA per ml.

The purity of the DNA was calculated by taking the ratio of A_{260}/A_{280}, where A is the absorbance.

Large subunit ribosomal gene sequening and analysis

The fungus was identified using a Microseq D2, large subunit (LSU) rDNA fungal sequencing kit ABI (Applied Biosystems, Foster City, CA).

The large subunit ribosomal gene (~300 bp) was amplified using the PCR master mix. A 50 µl reaction was set up in a thermal cycler (Eppendorf, Germany). The thermal cycling conditions were 96 for 10 sec., 30 cycles of 94 °C for 30 sec., 55 °C for 30 sec. and 72 °C for 30 sec. followed by a final extension at 72 °C for 5 min. 5 µl of the PCR product was analyzed on a 1% (w/v) agarose gel. The remaining product was purified by Microcon – 100 column (Millipore, USA) filtration as per the instructions of the manufacturer. 40-60 ng of the product was used as template to prepare the sequencing product with the Microseq D2 sequencing master mix. The product was sequenced on an ABI prism 310 genetic analyzer (ABI, USA) according to the standard procedure of the manufacturer.

The DNA sequences thus obtained were submitted to the ribosomal gene database (http://rdp.cme.msu.edu and http://ncbi.nim.nih.gov) and the sequences aligned to identify the fungus. A sample of the fungus has been deposited in the Repository at the Institute of Microbial Technology, Chandigarh, India (Microbial Type Culture Collection (MTCC), Accession No: 5124).

Optimization of medium composition for the optimal production of camptothecin

For the production of CPT by RJMEF001 culture, spore suspension (10^5 spores ml^{-1}) of the culture was inoculated in Erlenmeyer flasks (500 ml) with different

mycological broths (100 ml). Basic liquid media were Czapek, Malt extract, Molasses, Goos and Tschessch, Potato Dextrose, Ashner and Kohn, Leonine, Bianchi and Sabouraud supplemented with different salts or salts with trace elements and designated as M_1, M_3, M_6, M_7, M_9, M_{10}, M_{11}, M_{12} and M_{18} respectively throughout in the present study (Table 5). All media were adjusted to pH 7.0±0.2 except Sabouraud and Potato Dextrose broth where pH was maintained 5.6 and 6.0 respectively and were sterilized by autoclaving for 20 min at 121°C. The fungal cultures were incubated on a rotary shaker (220 rpm) at 28 ± 2°C for 4 days. Different carbon and nitrogen sources like glucose, dextrose, sucrose, maltose, starch, malt extract, yeast extract, peptone, molasses, corn steep liquor, beef extract, urea, ammonium oxalate and ammonium nitrate were added in order to find out the best nutrients combination for biomass, sporulation and CPT production. All the flasks were harvested after 4 days of incubation period. After filtration and washing the mycelia with demineralized water, the moisture content, dry cell weight and final pH were recorded. The fungal mycelia were extracted with $CHCl_3$: CH_3OH (4:1 v/v) solvent mixture. All the mycelial extracts were monitored on TLC plates run in $CHCl_3$: CH_3OH (9:1 v/v). The experiments were performed in triplicates and were repeated three times.

Growth and production kinetics of RJMEF001 and CPT in shaken flasks and in bioreactors

The organism used in the present study was grown in presterilized sabouraud broth with dextrose monohydrate (DMH) as sole carbon source (4%w/v) and peptone (1%w/v) as nitrogen source. DMH was autoclaved separately at 115°C for 10 min. Shake flask experiments were carried out in 500ml Erlenmeyer flasks containing 100ml of medium, agitated at 200 rpm on a rotary shaker at 28± 2°C. Large-scale cultivation of fungus was performed in 40 l bioreactor (New Brunswick Scientific USA). The working volume was kept at 18 l with an aeration rate of 1 volume per volume per minute (vvm), vessel pressure 0.2 kg cm^{-2}, temperature 28±2° C and agitation 220 rpm. In all the experiments, the initial spore count was maintained at 1x 10^5 spores ml^{-1} broth and the samples were drawn after 2 h inoculation taken as

zero h samples and subsequently every 24 h for a period of 216 h in shake flasks and 120 h in bioreactor experiments. Residual sugar, pH, dissolved oxygen and CPT production were determined.

The aeration rate was controlled by a mass flow controller (Brooks, US). Prior to the inoculation, the rate of air inlet was fixed at 1vvm and maintained at this level until the end of the culture period. Under these conditions an adequate mixing of the spore suspension (seed) was achieved throughout the culture period. Silicon oil (Hi-media, India) was added to avoid foam formation.

Wet weight of the mycelium pellet obtained after suction filtration of known volume of fungal culture was recorded. The mycelium pellet was then washed with several volumes of distilled water to remove the residual medium. A representative sample of pellet was dried at 50 ± 2° C for 14-18 h and dry weight per litre calculated. Sugar estimation was made by modified Cole's potassium ferricyanide methods (Coles, 1942).

Extraction and CPT measurements

Camptothecin was extracted from the fungal mycelia as described by Wani *et al.*, (1966). Mycelia and broth was separated by filtration (Fig. 14). Mycelia were thoroughly washed with sterile distilled water and homogenized in a cell disintegrator. Cell homogenates were extracted four times with equal volume of L.R. grade chloroform: methanol (4:1 v/v) mixture. Solvent was distilled off in a rotary evaporator leaving behind organic residue.

Thin layer chromatographic analysis

The residue on application on silica gel TLC plates (Merk K GaA, 64271 Darmstadt, Germany) run in (9:1 v/v) chloroform: methanol solvent system exhibited spots which were super imposable with the standard CPT. The spots were detected under UV at λ_{max} = 256 nm. The rest of the residue was used for quantification of CPT by LC-MS/MS.

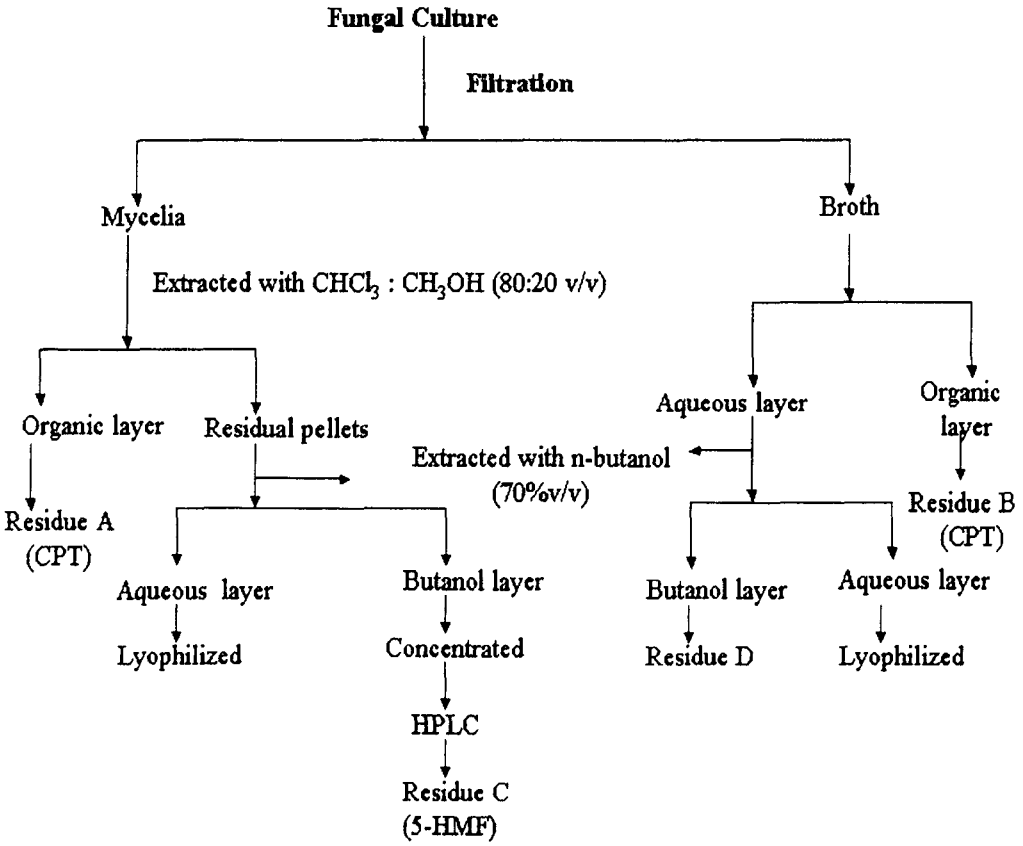

Fig. 14: Flow sheet showing the downstreaming process of fungal mycelia and broth

Examination of the sterile and spent medium for presence of Camptothecin

The sterile and spent medium was extracted following exactly the same procedure as with that of the fungal culture. LC-MS/MS comparisons demonstrated that the medium did not contain the fungal metabolite CPT but traces of camptothecin were detected in spent medium.

Effect of precursor molecules on the yield of camptothecin by *Entrophospora infrequens*

The fungal culture was separately grown in 500 ml Erlenmeyer flasks containing 100 ml Sabouraud broth, each supplemented with different precursor molecules. The incubation temperature was maintained at 28±2° C in a rotary shaker. Fungal culture was fed with precursors namely tryptophan, tryptoamine, geraniol, citral, mevalonic acid and leucine. The experiment setup was similar for the elicitation but in addition to that, precursors were fed either alone or in combinations (tryptophan and geraniol, tryptophan and citral, tryptophan and mevalonic acid, tryptophan and leucine). Stock solutions were prepared for each compound and pH was adjusted to 5.6 before adding to the culture at 0.01 M final concentration except for leucine whose concentration was adjusted at 0.025 M. The samples were taken after 96 h. The CPT was extracted with $CHCl_3$: MeOH (4:1v/v) mixture as described above. For the comparison of cell associated CPT content in fungal culture, the standard CPT was similarly subjected to HPLC analysis. The amount of CPT in each sample was determined by HPLC performed on a reverse phase column (RP-18, 2 mm i.d., .length 150 mm, and particle size 3 µm). The premixed mobile phase acetonitrile and water (25: 75 v/v) was run at flow rate of 1 ml min^{-1}. 10 µl of sample was injected in $CHCl_3$: MeOH (9:1 v/v). The CPT in the extract was identified by comparing its retention time with that of standard CPT isolated from *Nothapodytes foetida* (Puri *et al.*, 2005a) and authentic CPT.

Growth and production kinetics of fungal isolate under stationary conditions

The endophytic fungus *Entrophospora infrequens* was grown in 500 ml Erlenmeyer flasks, each containing 100 ml liquid sabouraud broth (Initial pH 5.6) for 35 days (28±2° C) unshaken. Spore suspension with initial count $1x10^6$ spores ml^{-1} was used as inoculum. Sampling was done at regular time intervals (every 7^{th} day). Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile distilled water, weighed and homogenized in a cell disintegrator as described above in the present study. Both cell homogenates and cell free broth of each culture was extracted and quantified by modified method of Hengel *et al.*, (1992) as indicated in the present study. Uninoculated control samples were also processed by usual procedures. The experiment was repeated thrice and the data presented as the mean value for each incubation time with standard deviation

Solid-state fermentation for the Production of CPT by the culture of *Entrophospora infrequens*

Solid -state fermentation experiments were carried out on moist wheat bran and wheat bran containing dextrose 40 gl^{-1}, peptone 10 gl^{-1}. The sabouraud broth and wheat bran were autoclaved at 121°C separately for 35 min and 15 min respectively. After autoclaving, the wheat bran was mixed with Sabouraud broth aseptically. After cooling, the medium was inoculated with a concentrated spore suspension (10-day old spores, $1x10^6$ spores/g dry matter). Initial pH and moisture content were 5.6 and 70% respectively. Incubation was carried out at 28±2° C for 28 days. Sampling was done at regular time intervals (every 7^{th} day). Fermented material was mixed with equal volume of sterile distilled water and homogenized in a cell disintegrator. The liquid extract was filtered (Whatman no.1) and extracted by normal procedure as mentioned above. Uninoculated control samples were also processed by usual procedures.

Results

Isolation and standardization of the culture of *Entrophospora infrequens* (RJMEF001) from inner bark of *N.foetida* plant

N. foetida (*Mappia foetida*; Family: Icacinaceae) was chosen as a source plant for isolating the endophyte, since this plant grows in unexplored environments in the high altitude ranges of the Himalayas where there is a possibility of mutualistic interactions between different groups of organisms. Earlier studies had indicated that virgin environments favour such interactions (Arnold *et al.*, 2000). The *N. foetida* plant has been reported to be the vital natural source of the camptothecinoids. The twigs of *N. foetida* plant were cut into small pieces, surface sterilized, rinsed with sterilized distilled water and inner tissues were isolated and placed on aqueous agar in Petriplates. At 28 ± 2° C, growth was found to be initiated. The tips of fungal hyphae were placed on mycological medium to obtain pure fungal culture, which was then transferred to a number of solid and liquid media, which supported the fungal growth. There was considerable variation in colony morphology between isolates, particularly in the shape of the colonies and the amount of aerial mycelium. Many colonies were bran like or convoluted, and / or crusted; aerial mycelium varied from dense cottony to sparse. Nearly all isolates were white, in the beginning but some turned pale brown. Most of the isolates did not sporulate on solid surface and in broth culture. Delayed sporulation was observed in twenty-two isolates out of fifty-two. However, specific sporulation medium was used for early and profuse sporulation. To establish the *de novo* production of camptothecin and camptothecinoids by the isolated fungus, the growing mycelium was serially transferred several times to fresh mycological agar to eliminate the possibility of the fungal hyphae carrying even minute amounts of camptothecin as contaminant from the source plant material.

Morphological characteristics of the fungus and identification

The endophytic fungus, isolated from the inner bark of *Nothapodytes foetida,* was investigated (Fig.15) on the basis of morphological characteristics and fungus ribotyping. The fungus grows on many semi-synthetic growth media at 28±2 °C,

covering plates with its mycelium in 10-12 days. Microscopic slides were prepared, stained by Lectophenol cotton blue and were examined microscopically (Olympus). The fungus has small hyphae which average 2-3μm in diameter (Fig. 16 a, b). The mycelia are branched, aseptate, ribbon shaped and multinucleate. Sporangiophores are long, unbranched, and wide and terminate into a sporangium (Fig. 16c). Sporangium is oval in shape and bear spores. The spores are round to oval in shape. The outer surface of spores bears prominent striations (Fig. 17). After 24 h of growth in Sabouraud Dextrose broth (with constant shaking) at 28° C, mycelial biomass could be collected in grams quantities. This collected biomass fraction was used further for DNA isolation, and the pellet isolated using TES buffer was vacuum dried and dissolved in TE buffer.

The dendrogram showing the phylogenetic tree constructed on the basis of rDNA sequences of the fungal strains nearest to it in the databases is given in the Fig. 18. This clearly shows that the strain is more close to *Entrophospora infrequens* (>98%) than the other close taxa e.g., *Rhizopus oryzae* strains UWFP 973 and 846 (>97%). On the basis of the morphological features, growth behaviour as well as more percent homology to *Entrophospora infrequens* it seems to be a strain of the former taxa. The fungal strain has been deposited at MTCC, Chandigarh, India under accession No. 5124 and a PCT application filed (Puri *et al.*, 2002).

Effect of different media on growth and CPT production

Various growth media were tried at shake flask level to find the appropriate media for the optimum growth of the organism and the camptothecin production (Table 5). High CPT production of 503.07 ± 25.88 μg/100 g dry cell mass was observed when *E. infrequens*- RJMEF001 was grown in Sabouraud broth (M_{18}) with maximum dry biomass of 14.24 ± 1.37 gl^{-1}. The camptothecin production in Sabouraud broth supplemented with trace elements magnesium sulphate (0.5 gl^{-1}) and potassium dihydrogen orthophosphate (1.0 gl^{-1}), Goos and Tschessch, Leonine and molasses liquid medium was 489.42 ± 19.89 μg, 326.42 ± 36.55 μg, 200.34 ± 9.95 μg and 230.16 ± 25.88 μg/100 g dry cell mass with cell mass of 14.54 ± 1.0 gl^{-1}, 12.34 ± 0.9 gl^{-1}, 15.7 ± 1.0 gl^{-1} and 8.09 ± 1.1 gl^{-1} respectively at 96 h of

incubation time. In potato dextrose broth only traces of CPT were detected. Lowest CPT production was observed with Leonine medium. There was no CPT formation in malt extract (M_3, M_{15}, M_{16}), Czapek (M_1, M_2, M_{13}, M_{14}), Ashner and Kohn and Bianchi liquid broths. The highest cell mass of 28.48 ± 1.08 gl^{-1} was recorded in Czapek (M_{14}) with carbon source Dextrose (3% w/v) and nitrogen (0.3% w/v) followed by 28.17 ± 1.46 gl^{-1} and lowest cell mass of 1.84 gl^{-1} in osmotic stress broth with 10% NaCl.

Effect of carbon and nitrogen sources on the growth and CPT production

RJMEF001 was cultured in the medium containing different carbon, organic and inorganic nitrogen sources to find the conditions leading to the highest production of the camptothecin. *E. infrequens* grown at shake flask level showed a significant increase in CPT production 503.07 ± 25.88µg/100g dry cell mass in sabouraud broth when concentration of 1% (w/v) peptone was incorporated into the medium (Table 5) with other nitrogen sources like malt extract, corn steep liquor, beef extract and yeast extract did not show significant increase in CPT production, similarly inorganic nitrogen sources like urea, ammonium oxalate also failed to have any influence on the CPT production.

Various sugars when examined as carbon sources at different concentrations (0.2-4%, w/v) failed to show any increase in CPT production. The presence of carbon sources like sucrose and maltose did not produce any significant change in the CPT production profile. There was appreciable decrease in cell mass (3.0 ± 0.9 gl^{-1}) production when culture was grown in the presence of triple sugars like, dextrose, sucrose and starch. Eventhough an increase in the cell mass by nine fold was observed when grown in Czapek media. Presence of starch ceased the CPT production and also decreased cell mass production (Table 5).

Shake flask experiments for the production of camptothecin

Shake flask experiments with the fungal culture were conducted to evaluate the optimal conditions for growth and its capacity to produce secondary metabolites.

The inoculation conditions and the fungal culture in 500 ml Erlenmeyer flasks are summarized in the Table 6. The growth period of the culture extended in shake flasks to 6 days of incubation period and peaked at 7th day (Fig.19a). No sporulation was observed in submerged culture. The fungal mass of 2.3 ± 0.43 gl^{-1} was measured at 24 h and a maximum production of 28.11 ± 1.92 gl^{-1} on day 7. As far as the CPT accumulation in the cell mass is concerned, it started right at 24 h through to 96 h (Fig.19b) of incubation time, thereafter a decline in the content was observed. The highest cell associated CPT content was found to be 0.575.± 0.031 mg/100 g dry weight of mycelia. Data represents mean of three replicates and the experiment was repeated six times to get reproducible results. Production of secondary metabolite (CPT) precedes growth in shake flask.

Growth and CPT production in bioreactors

Different batches of bioreactors from 5 litres (airlift Lh- model 5-2000 series, UK) to 18 litres (NBS-New Brunswick Scientific USA) capacity were run for the optimization of fermentation conditions for the maximum growth of biomass and production of camptothecin. These bioreactors were run under similar and simulated conditions and also under different conditions for optimization of the fermentation parameters for maximum CPT production. The conditions found to be optimal for the growth of *Entrophospora infrequens* and production of secondary metabolite of fungus are summarized in Table 6. Growth of mycelium begins 2-3 h after the inoculation and peaks at 96 h in 18 l bioreactor, there after a gradual fall in the biomass production was observed, which might be due to partial lysis of cells after attaining stationary phase. Temperature of 28± 2°C was found to be congenial for the growth of mycelium and production of camptothecin. The pH of the medium in the bioreactor was initially recorded to be 5.6 which started decreasing right from day one and reaches the minimum of 2.5 at 120 h of fermentation .At the start of the stationary phase (96 h), 35.58 ± 3.45g dry weight per litre (Fig. 20a) was obtained. The pH of the medium falls gradually as the fermentation time increases and maximum camptothecin was recorded at pH 3.5 (Fig. 20b). The Dissolved oxygen decreased to 56% saturation at the end of fermentation. The

residual sugar concentration of 1% was observed at the time of maximum production of camptothecin (Fig. 20c). There was a steady increase in the growth of mycelium from day 1 to day 4 in the bioreactor but maximum camptothecin was recorded at 48 h (Fig. 20 d) of fermentation which indicates that production of camptothecin started right after the first day of incubation when sugar percentage also started declining, where as in the shake flask the maximum Camptothecin was recorded at 96 h of fermentation. The production of the CPT by the culture started declining after 4 days and there was almost stationary phase of CPT production after 7 days of fermentation in shake flasks. The highest CPT content in the bioreactor culture (4.96 ± 0.73mg/100g dry mycelium at 48 h) is several folds higher than the respective content in the shake flask culture (0.575.± 0.031mg/100g dry mycelium at 96 h). As it is generally possible to achieve an adequate aeration and mixing in bioreactor cultures, the higher CPT production yields could be the result of an appropriate combination of all the above given factors.

Stimulation of the production of CPT by biogenetic precursors and elicitors in fungal culture

Sabouraud broth containing geraniol, citral precursors and tryptophan in combination with geraniol and citral showed good cell growth without production of desired metabolite CPT. But CPT production was observed when the fungal culture was grown in sabouraud broth supplemented with tryptophan, tryptoamine, mevalonic acid and tryptophan in combination with leucine. However, fungal culture grown in Sabouraud broth supplemented with different precursors and combination of precursors showed no difference in morphology i.e. only clump formation was observed under submerged culture conditions and final pH was found acidic, which decreased from 5.6 to minimum of 2.5. From Table 7 statistical analysis gives less dry biomass production in tryptophan and leucine as compared to the other treatments. Whereas the CPT production with this kind was also significantly different from other treatments. The idea of using l-leucine was taken from the studies in the biosynthesis of lophocerine where two alternative routes for the biosythesis have been successfully elucidated. Lophocerine is an isoprenyl

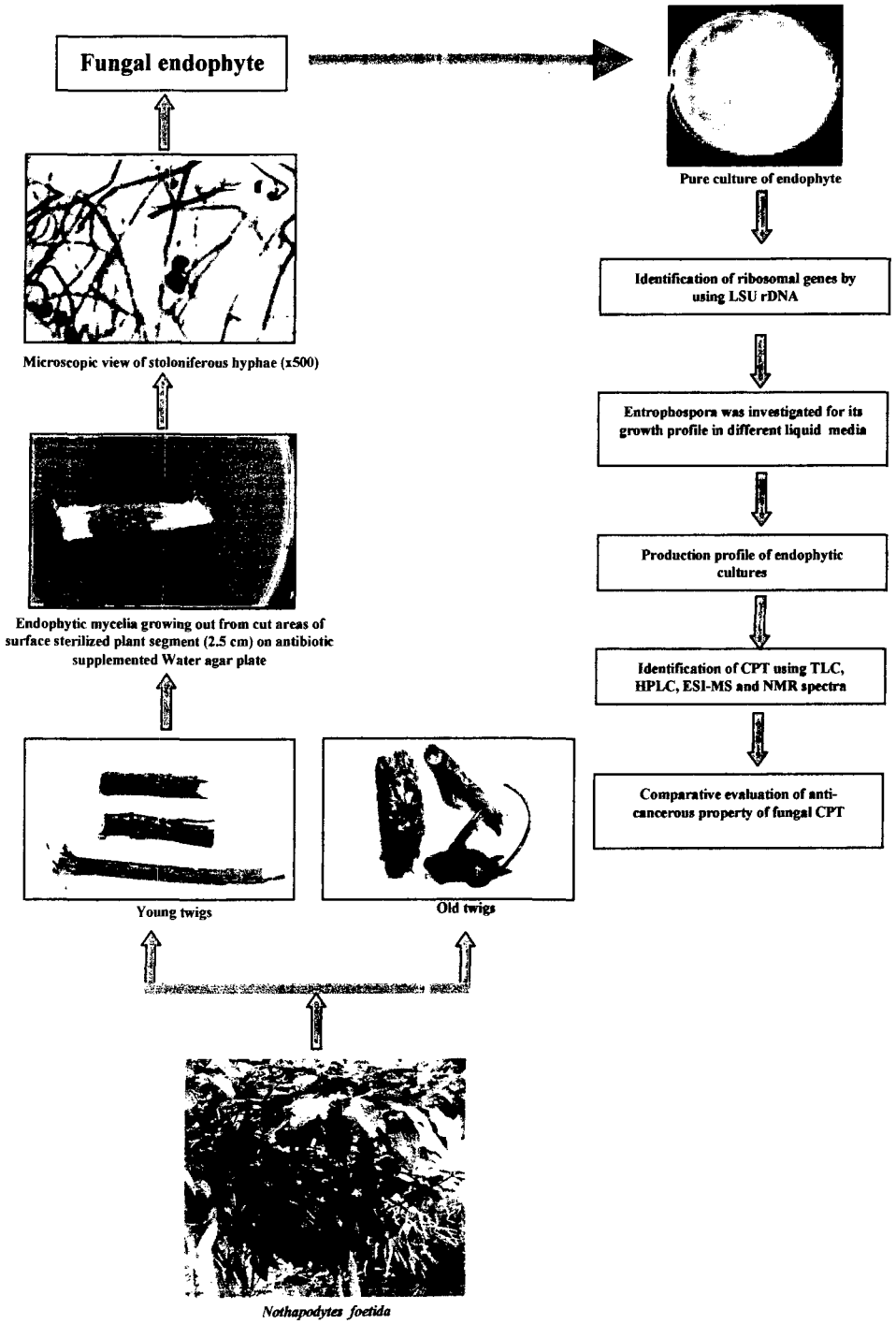

Fig. 15: Schematic representation of the experimental strategy adopted for isolation, identification and maintenance of the culture of an endophyte associated with *Nothapodytes foetida* (approx. 400 m altitude)

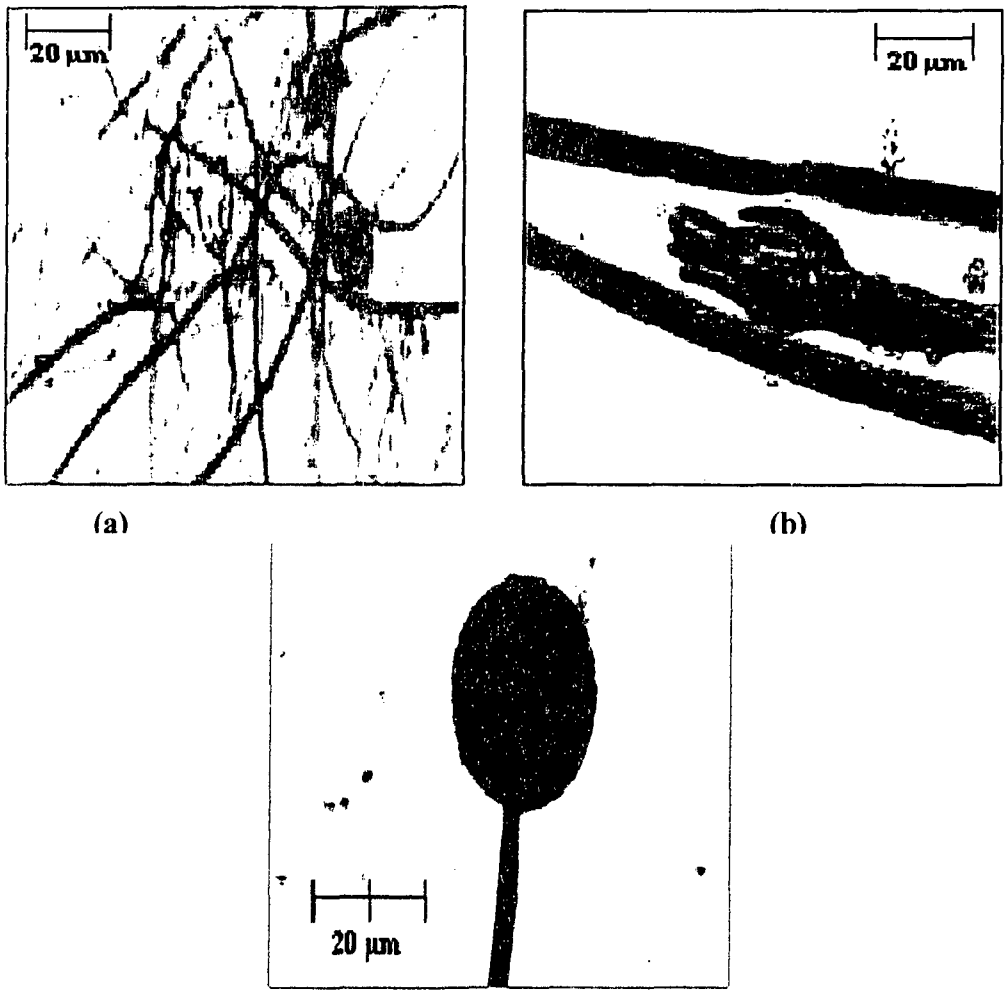

Fig. 16:(a) Microscopic view of horizontally growing unbranched stoloniferous hyphae (x 500); (b) Microscopic view of horizontally growing unbranched stoloniferous hyphae (x 1000); (c) Microscopic view of the young sporangium of endophytic fungus (x 1000)

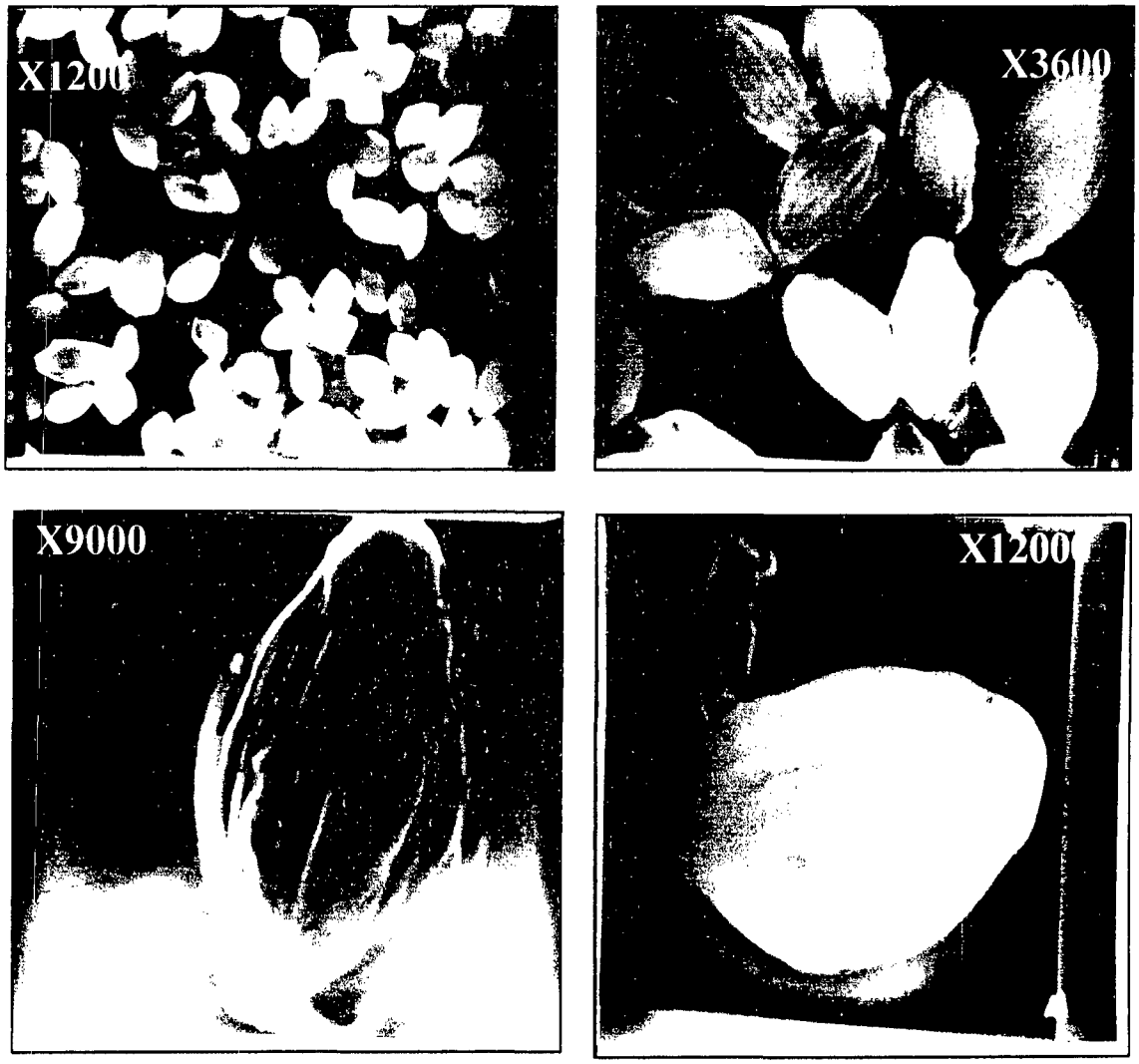

Fig. 17: Scanning electron micrographs depicting detailed surface ultra structural characteristics of spores of *Entrophospora infrequens*

GGAAAGAAAAAGACTTTGAAAAGAGAGTAAACAGTATGTGAAATTGTTAAAAG
GGAACCGTTTGGAGCCAGACTGGCTTGTCTGTAATCATCTAGGCTTCGGCCTGG
ATGCACTTGCAGGCTATGCCTGCCAACGACAATTTGACTTGAGGGAAAAAACTA
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TGGATTGAGGAACGCAGCGAATCTTATTGGCGAGTTTTCCAGGAAGGTTTTCTG
AGGTACTACGGTATCAAGGTTGATCTTTTTGGTTATACTTCTATTCGCTTAGGTT
GTTGGCTTAATGACTCTAAATGACCCGTCTTGA

Sequence homology:

Entrophospora infrequens isolate SSA14	>98%
Entrophospora infrequens isolate SSA12	>98%
Entrophospora infrequens isolate SSB05	>98%
Rhizopus oryzae NRRL28631	>97%

Fig. 18: (a) Large subunit ribosomal gene sequence of isolate RJEF001 and homology with other strains

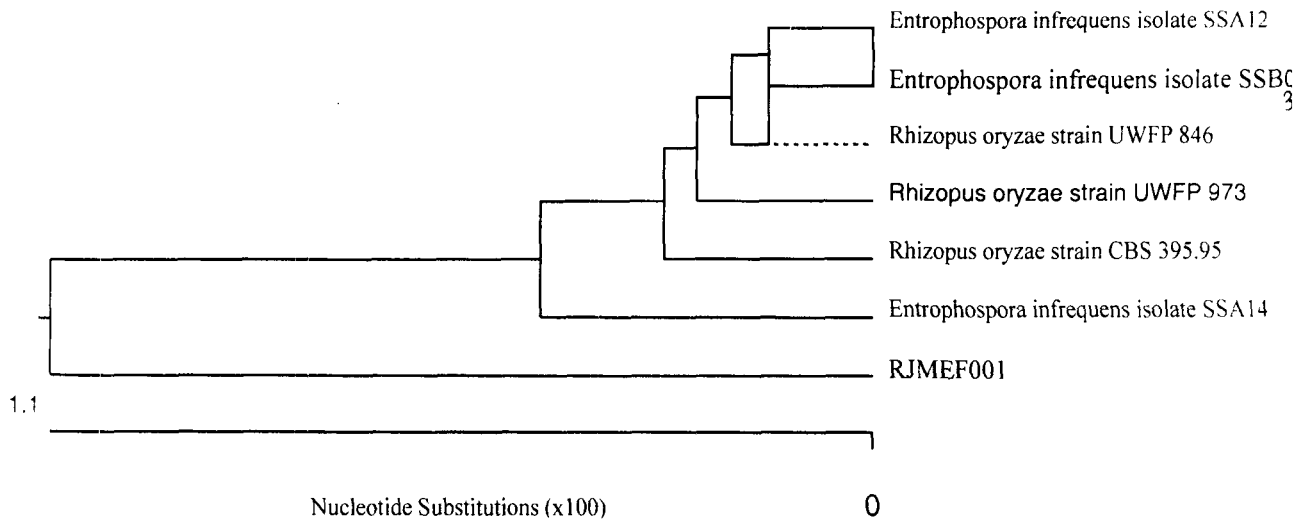


Fig. 18:(b) Dendrogram showing the phylogenetic position of isolate RJMEF001 as obtained by alignment of large subunit ribosomal gene sequences using Clustal W software

Table 5: Biomass and production of CPT by RJMEF001 isolate under submerged conditions on various growth media

Media (M)	Dry Biomass (gl^{-1})		CPT (µg/100g Dry Cell Mass)	
	$\overline{X}$	S	$\overline{X}$	S
M_1	27.58	1.28	ND	ND
M_2	27.13	2.01	ND	ND
M_3	13.34	0.95	ND	ND
M_4	22.30	1.95	ND	ND
M_5	28.17	1.46	ND	ND
M_6	8.09	1.14	230.16	25.88
M_7	12.34	0.97	326.42	36.55
M_8	15.65	1.20	ND	ND
M_9	18.17	1.42	ND	ND
M_{10}	24.45	1.65	ND	ND
M_{11}	15.71	1.05	200.34	19.95
M_{12}	3.00	0.90	ND	ND
M_{13}	25.66	1.36	ND	ND
M_{14}	28.48	1.08	ND	ND
M_{15}	1.84	0.06	ND	ND
M_{16}	2.45	0.32	ND	ND
M_{17}	27.71	1.26	ND	ND
M_{18}	14.24	1.37	503.07	25.88
M_{19}	14.54	1.04	489.42	19.87

Values are triplicate determinations and SD of experiments

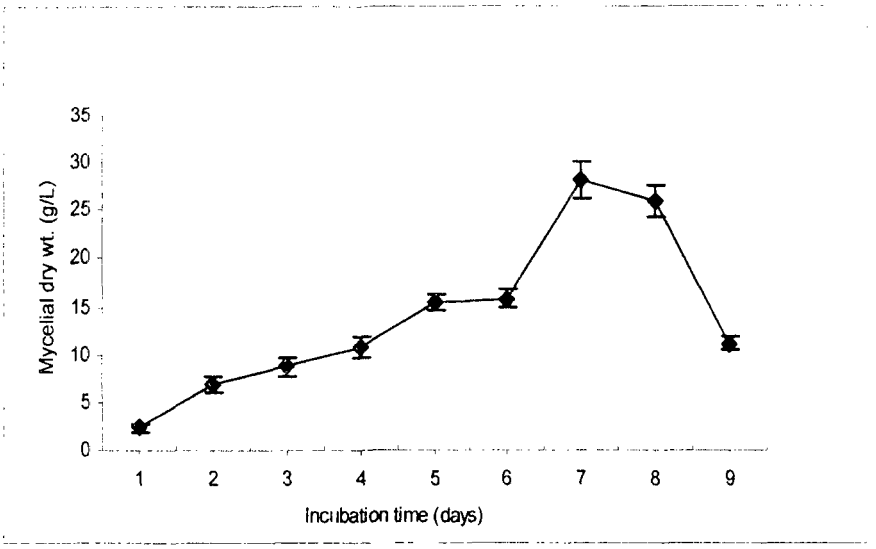
Where $\overline{X}$ = mean determinative

S= Standard deviation of mean

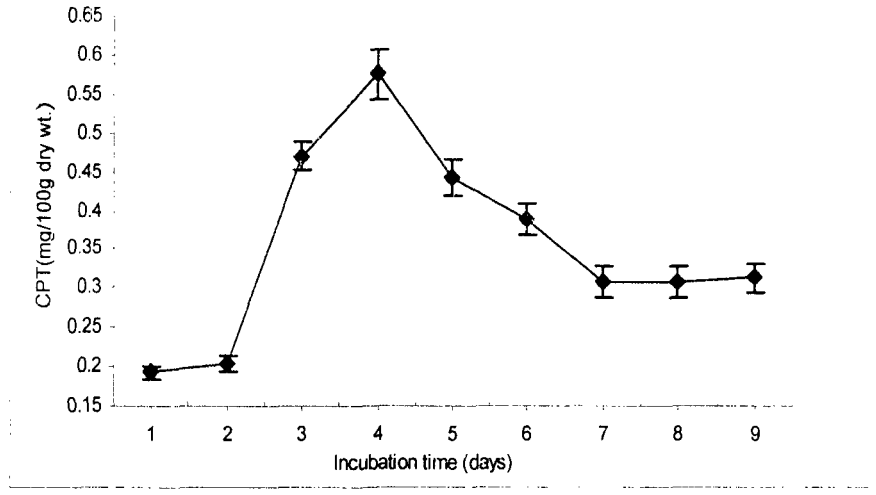
ND- Not Detected

Table 6: **Cultivation parameters for the *Entrophospora infrequens* culture in shake flask and an 18l bio-reactor**

Cultivation system		**Shake flask**	**Bio-reactor**
	Inoculum	Spores (10^5 spores ml^{-1})	Spores (10^5 spores ml^{-1})
	Medium	Sabouraud	Sabouraud
	Cultivation time	216 h	120 h
Parameters	**Fermentation modus**	Batch	Batch
	Working Volume	100 ml	5-18 l
	Temperature	28± 2°C	28± 2°C
	Vessel pressure	-	0.2 kg cm^{-2}
	Aeration rate	-	1vvm
	Antifoam	-	Silicon oil
	rpm	200-220	200-220



(a)



(b)

Fig. 19:(a) Growth profile of *Entrophospora infrequens* (b) Production profile of *Entrophospora infrequens in* shake flask. Data represents the mean of three replicates ± SD and point to point comparison indicates biomass production at day 7and CPT production at day 4 statistically significant as determined by unpaired student's t-test

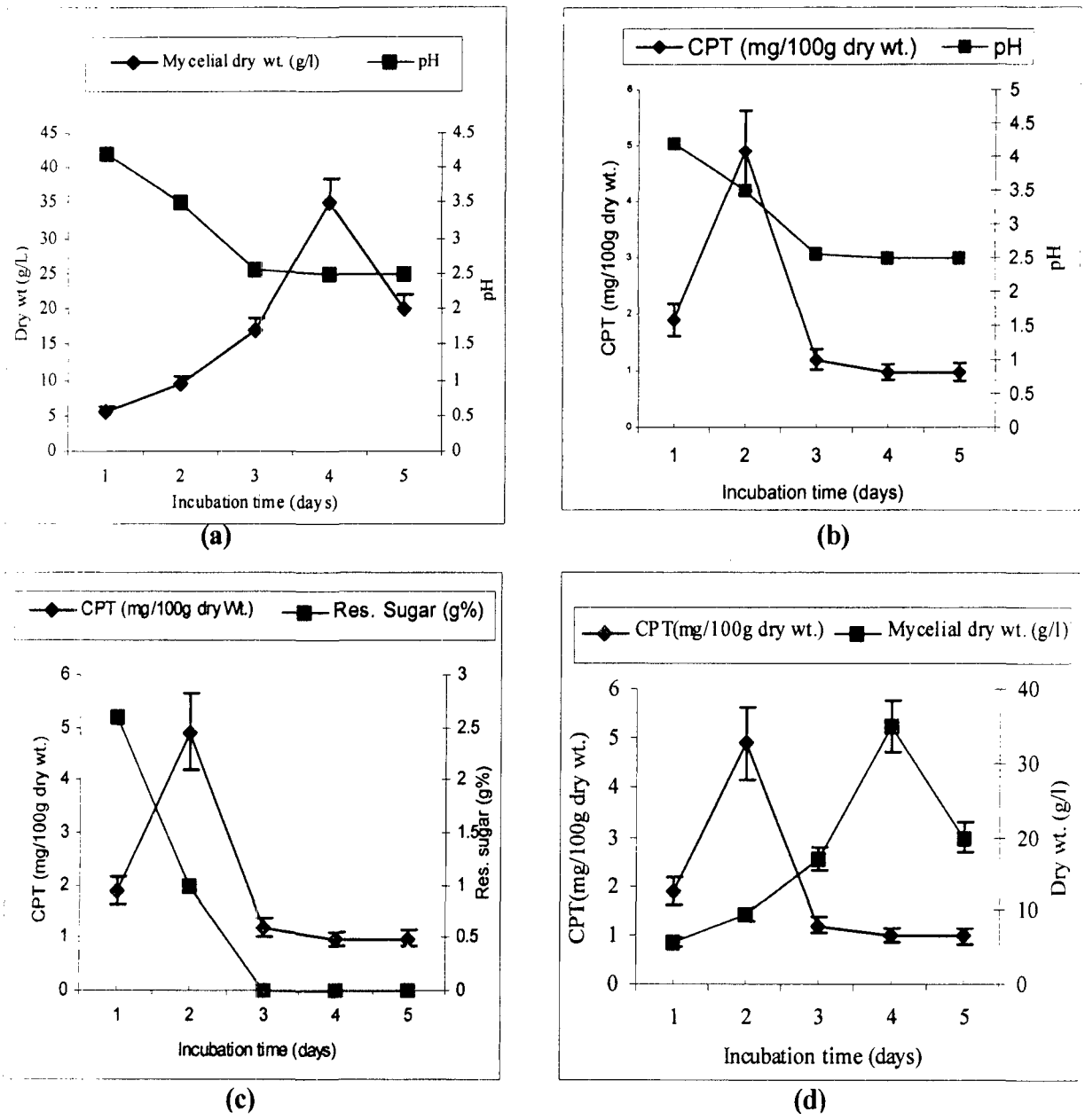


Fig. 20: Effect of incubation period on (a) Biomass and pH (b) CPT and pH (c) CPT and residual sugar (d) Biomass and CPT production by *Entrophospora infrequens* in 18 L bioreactor. Data represents the mean of three replicates ± SD and point to point comparison indicates biomass production at day 4 and CPT production at day 2 statistically significant as determined by unpaired student's t-test

Table 7: CPT production by endophytic fungal culture in Sabouraud broth supplemented with different precursors under submerged conditions

Precursor	Conc. (M)	Dry cell mass (gl^{-1})		CPT (µg/100 g dry wt.)	
		$\overline{X}$	S	$\overline{X}$	S
Geraniol and Tryptophan	0.01	18.85	1.15	ND	ND
Geraniol	0.01	17.30	1.05	ND	ND
Citral	0.01	16.98	0.92	ND	ND
Citral and Tryptophan	0.01	18.98	1.15	ND	ND
Tryptophan	0.01	19.90	1.15	871.58	86.2
Tryptoamine	0.01	19.00	1.10	797.69	75.3
Tryptophan and Mevalonic acid	0.01	16.0	0.92	305.02	30.6
Tryptophan and Leucine	0.01 + 0.025	11.0	3.46	993.68	99.3
Blank	-	14.0	1.37	491.08	49.1

Values the mean $\pm$ SD of 3 replicate determinations

Where $\overline{X}$ = mean determinative

S= Standard deviation of mean

ND- Not Detected

alkaloid. It has been clearly observed that leucine can be substitute for the isoprenyl pyrophosphate, which in turn is generated from mevalonic acid (Dewick, 2002).

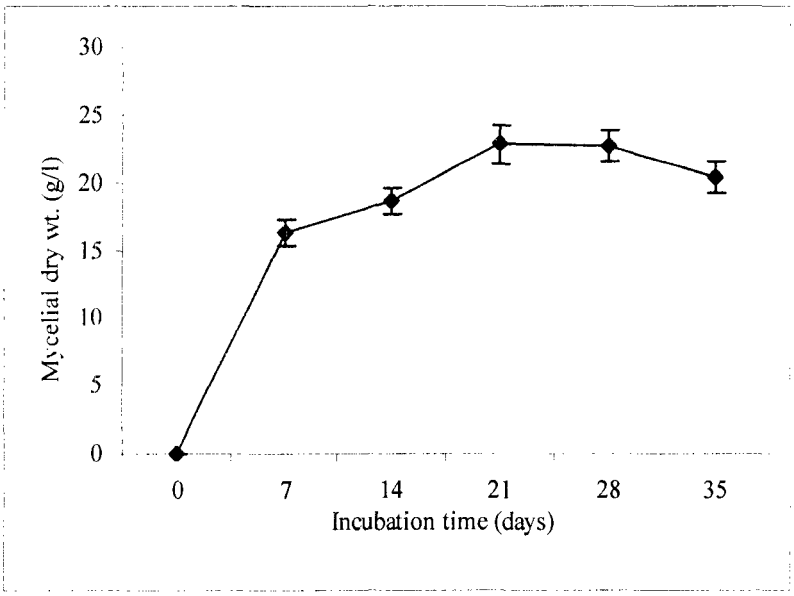
Growth and production kinetics of fungal isolate under stationary conditions

The mycelial mat formation started after 3 days of incubation time and complete mycelial mat formation was observed at 8th day. The maximum mycelium was found to be 16.24± 0.94 gl^{-1} at 7th day, which increased during the course of incubation period and peaked (22.79± 0.80gl^{-1}) at 21 days. The pH of the medium decreased from 5.6 to minimum of 4.5 (Fig. 21a). CPT was followed as a function of time in still culture. The mycelia and broth extracts of fungus were found to accumulate CPT in trace amounts after 7 days of incubation period. The camptothecin peaked at 21 days of incubation and rapidly declined after 28 days (Fig. 21b), however the biomass continued to grow. The maximum yield of CPT was found to be 3.37 ± 0.44 mg per 100g dry weight and was 250 ± 20 µgl^{-1} of broth. The temperature of 28±2° C was maintained during the course of study. No CPT formation was observed in uninoculated culture broths those have been extracted with Chloroform: Methanol (4:1v/v) solvent mixture and formation of the same was also not observed in inoculated, extracted and processed culture broths at zero time. The experiment was carried out three times in replicates of three to confirm the results.

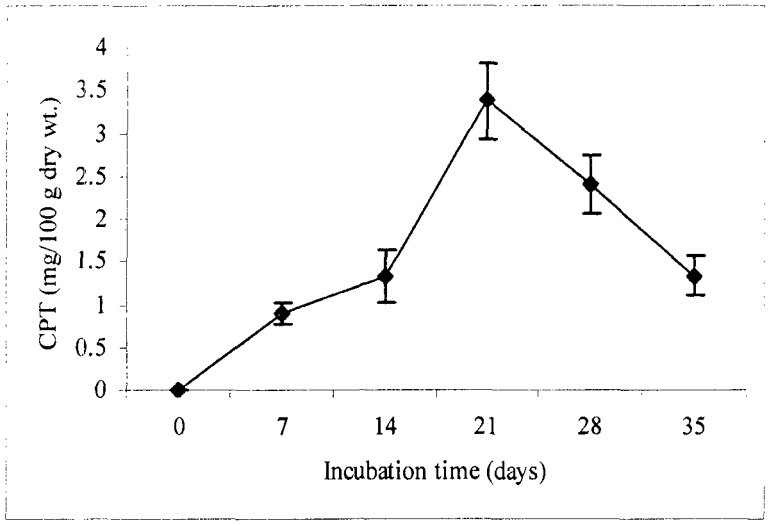
Solid-state fermentation

Solid state fermentation (SSF) using inert supports impregnated with chemically defined liquid media has several potential applications in both studies and in the industrial production of high value products, such as metabolites, biological control agents and enzymes. In the present study, the solid-state fermentation was carried out on moist wheat bran and wheat bran supplemented with Sabouraud broth. The fungal mycelia emerged out after 2 days of incubation time and showed complete white cottony mat formation after 4 days of incubation period. The initiation of sporulation was observed at 8th day of incubation. The flasks were harvested at zero, 7, 14, 21 and 28 days and processed. The CPT production started at 7th day

and the maximum yield of 200.33 ± 19.90 µg/100 g dry weight and 390.78 ± 20.10 µg/100 g dry weight on wheat bran and wheat bran supplemented with sabouraud broth respectively was observed at 21 days (Fig. 22 a), thereafter decline in the production was observed. However the maximum biomass of 13.80 ± 1.04 gl^{-1} and 14.10 ± 1.09 gl^{-1} (Fig. 22 b) on wheat bran and wheat bran supplemented with sabouraud broth respectively was observed at 14 days.

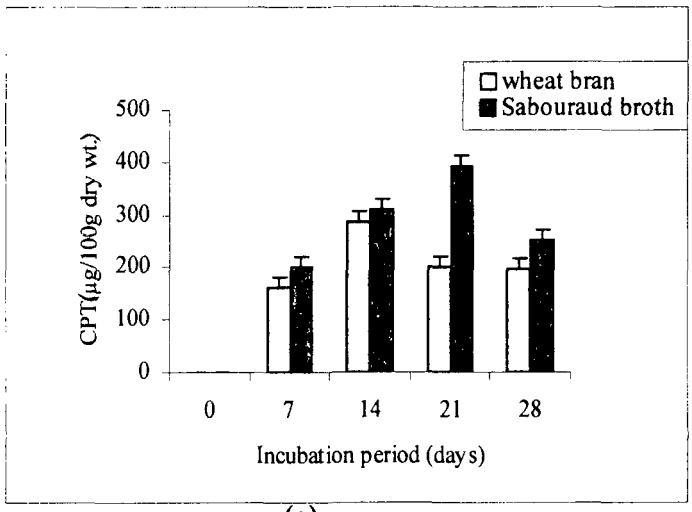


(a)

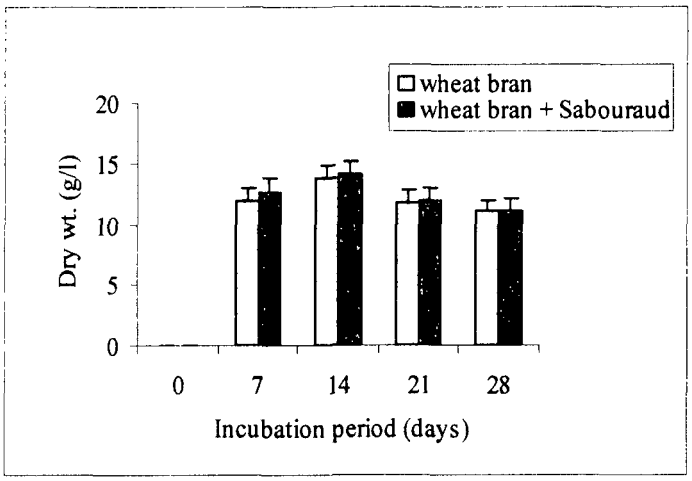


(b)

Fig. 21:(a) Mycelial dry wt. and (b) CPT production by *Entrophospora infrequens* as a function of time in still culture. CPT was isolated and quantified and the mycelium was harvested, dried and weighed. Each weekly point is the mean of four determinations ± SD



(a)



(b)

Fig. 22: (a) Production profile of *Entrophospora infrequens* (b) Growth profile of *Entrophospora infrequens* on wheat bran and wheat bran supplemented with Sabouraud broth. Data represents mean of three replicates ± SD.

CHAPTER 4

PART I

CHEMICAL STUDIES ON *N. FOETIDA* ASSOCIATED ENDOPHYTIC FUNGUS

Materials and Methods

Melting points were determined with a Buchi melting point apparatus. (Model B-545) and are uncorrected. Infrared spectra were obtained on a Hitachi 270-30 spectrophotometer in KBr pellets. ^{1}HNMR (500 MHz), ^{13}CNMR (500 MHz) and 2DNMR spectra were determined on a Bruker Avance-500 spectrometer. FAB-MS was recorded on a JEOL SX 102/DA-6000 mass spectrometer. Optical rotation was obtained on a Perkin Elmer 241 polarimeter. Elemental analytical data was recorded on Carlo Erba, Model 1106, elemental analyzer. Column chromatography was carried out using SiO_2 gel (60-120 mesh, Merck). Spots on TLC were visualized by spraying with 1% Cericammonium sulphate in 30% aqueous H_2SO_4 followed by heating the plate at 105°C for 15 minutes.

Isolation of Camptothecin from endophytic fungus (RJMEF001)

900 g of partially dried fungus was extracted with a mixture of chloroform and methanol (80:20) in a separating funnel repeatedly (4 times) and the pooled extract was distilled to yield 15 g of the residue.15g of residue obtained from extraction of mycelia was subjected to column chromatography over silica gel column (600 g, 60-120 mesh) eluted with hexane followed by benzene-ethyl acetate mixtures of five increasing polarity profiles (95: 5, 90:10, 80: 20, 50:50, 10:90). The column was further eluted with ethyl acetate-chloroform (90:10, 80:20, 70:30, 60:40, 50:50, 20:80) and chloroform-methanol (90:10, 80:20, 50:50) and finally drained with 100% methanol. Fractions of each distinct polarity were pooled to obtain 18 fractions and subjected to TLC and HPLC. Positive fractions showing bluish fluorescent spot under UV (λ_{max} = 256 nm) with the same Rf as that of standard CPT were pooled and concentrated to give 150 mg of material with some impurities. This mixture was rechromatographed further on silica gel (20 g, 60-120 mesh). Elution was started with chloroform, gradually increasing the percentage of methanol in chloroform. The fractions eluted in 1- 5% methanol in chloroform showed same Rf as that of standard CPT. These fractions were pooled and crystallized (70 mg). The compound was identified as CPT by spectral analysis and m-mp (275-277° C).

High Performance Liquid Chromatography (HPLC)

HPLC separation was performed using Luna RP-18 column (2 mm i.d., length 150 mm, and particle size 3 μm) and a safety guard (Phenomenex, Torrance, CA, USA) at 30°C. The mobile phase water (A) and acetonitrile (B) was run in the following manner (0-5 min 90% A and 10 % B, 5-20 min 40 % A and 60 % B, 20-30 min 2% A and 98% B, 30-32 min 2% A and 98 % B and 35-37 min 90 % A and 10% B v/v) at a flow rate of 200 μl min^{-1} detection: UV at $\lambda = 256$ nm, $t_{R_-} = 20.15$ min. 10 μl of sample was injected in $CHCl_3$: MeOH (4:1). Its purity was found to be 98. 5% (Fig. 23 a, b).

Low-resolution MS was performed by EI ionization (Finnigan-MAT 8000) at 70 eV, with a direct inlet probe at 252°C. A Finnigan TSQ 7000 with ESI ionization in the MS/MS mode was used. The optimal collision energy was determined by means of an ICL procedure controlling the automatic switching between different voltages, with a step size of 0.5 V/scan to 40 V. During this procedure, the analytes were injected via a Rheodyne valve with a 2 μl injection loop at a concentration of 10 μg ml^{-1}. A prescan voltage settling time of 20 min and 0.4 s for one complete cycle (four transitions) was used for selected reaction monitoring (SRM). HRMS was done using a JEOL JMS/SX 102 A FAB ion source (matrix, 3-nitrobenzyl alcohol; calibration, PEG 400; resolution, 10,000) and an Apex III FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) with a 7 T super conducting magnet. Positive ions were produced in an external Apollo electrospray ion source (Bruker Daltonics, Billerica, MA) with a flow rate of 2 ml min^{-1}. Infrared multiphoton dissociation (IRMPD) and activation of ions in the ICR trap were performed using a CO_2 J48-2 laser with 25 W maximum power output (Synrad, Mukilteo, WA). The optical rotation measurement was performed using a Perkin-Elmer 341 polarimeter with a tube of 10 cm cell path length using $CHCl_3$: MeOH (8:2) as solvent. CD was performed using a JASCO J715 spectropolarimeter, with a Hellma precision quartz glass Suprasil cuvette, a 1 mm light path length and $CHCl_3$: MeOH (4:1) as solvent. FTIR Bruker IFS (KBr) was used for recording the IR spectra. UV spectra were obtained using a Varian CARY 100 BIO, 1 cm cuvette

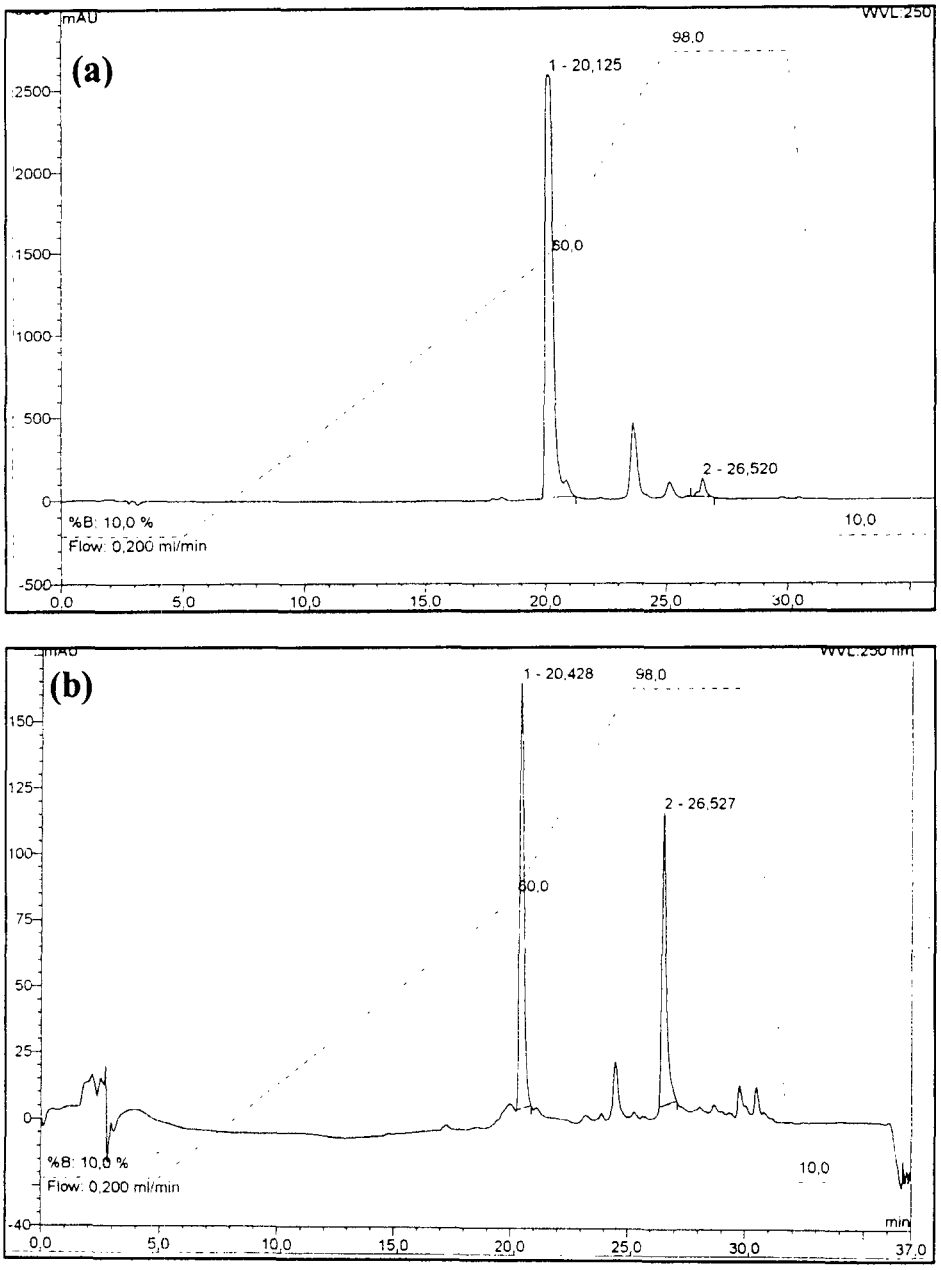


Fig. 23: HPLC profile of (a) Authentic Camptothecin (b) Fungal Camptothecin

and $CHCl_3$: MeOH (4:1) as solvent. For ^1H and ^{13}C NMR spectra, a Bruker AMX 600 instrument was used.

Quantification of camptothecin in *Entrophospora infrequens* by LC-MS/MS

Preparation of stock and standard solutions

Camptothecin stock solution (1 mg ml^{-1}) was prepared in HPLC grade chloroform: methanol (9:1) solvent mixture. The stock solution was stored in the refrigerator at 4 °C. From the stock solution the working solutions were prepared in the concentration range of 0.5 ng μl^{-1} to 5 ng μl^{-1} for LC-MS/MS analysis.

Instrumentation

Fungal culture was grown and processed as described earlier (Chapter-3) in surface culture studies. Both mycelial and broth extracts were used for quantification of CPT by LC/MS/MS.

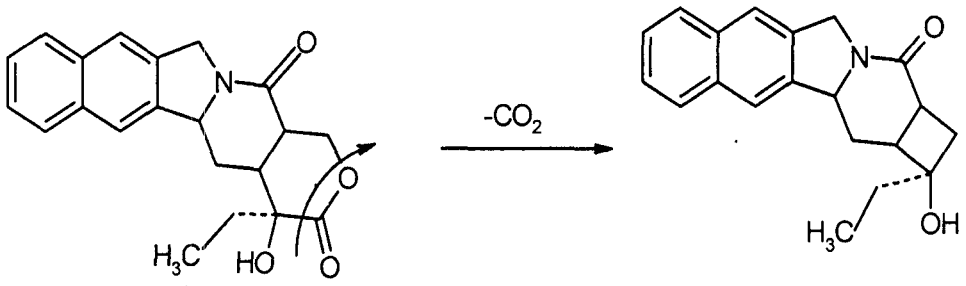
Samples were analyzed on an Agilent 1100 series HPLC system comprising binary HPLC pump, diode array absorbance detector, an autosampler, an online degasser and thermostatic column oven. Separations were carried out using 250 x 4.0 mm i.d, 5 µm, RP-18e column, (Merck, Germany). A gradient of water and acetonitrile at a flow rate of 0.5 ml min^{-1} was employed as the mobile phase. The gradient used started with 10% acetonitrile (5 min isocratic) and over a period of 35 minutes the percentage of acetonitrile was increased to 98% (10 min isocratic) and subsequently decreased again to 10% acetonitrile. The total analysis run time was 50 minutes. The LC column temperature was maintained at 30°C. After passing through the flow cell of DA detector the column eluate was directly transferred to MS detector without any split. The mass spectrometer was fitted with an electrospray interface. The entire interface parameters of LC/MS/MS studies were optimized by using the standard solution of CPT. The other parameters for LC-MS/MS analysis were set at dry gas flow of 11 l min^{-1}, nebulizer pressure 35 psi and drying gas temperature 320° C. The isolated peak width was taken as 0.8 *m/z* and fragmentation amplitude value was 2.40.

Mass spectrometry of Standard CPT and fungal extracts

A mass spectrum of CPT in methanol: chloroform [1:3] was recorded under ESI on a Bruker Ion Trap mass spectrometer in the +ve ion mode with a mass range from 50-800 a.m.u. The most intense peak in the mass spectrum corresponded to the $[M+H]^+$ ions of CPT at m/z 349 (Fig. 24). The sodium adduct of CPT was also formed and was visible in the mass spectrum at m/z 371 $[M+Na]^+$. The molecular ion peak at m/z 349 $[M+H]^+$ was taken for MS/MS studies (Lemoine *et al*; 1993). This molecular ion peak on isolation and fragmentation exhibited daughter ion peak (Scheme 1) at m/z 305 $[M^+ + H - 44]^+$ which was due to the loss of carbon dioxide (Fig. 24 and 25) moiety. This daughter ion peak at m/z 305 was taken up for quantification in a Single Reaction Monitoring System (SRM).

LC-UV (DAD)-MS/MS analysis

Using above conditions satisfactory results were obtained in the positive mode ESI-MS/MS. Fig. 26 shows the Total Ion Current (TIC) trace from SRM (a), LC-UV (DAD) (b) chromatogram (256nm) and LC-ES-MS/MS spectra (c) of CPT under the positive mode of electrospray ionisation. Fig. 27 shows the Total Ion Current Chromatogram (a) from SRM, and the LC-UV (DAD) chromatogram of CPT (b).



Camptothecin
m/z 349$[M+H]^+$

Daughter ion
m/z 305 $[M+H]^+$

Scheme 1. Proposed first fragmentation (MS^2) of Camptothecin

Quantification of CPT in fungal extracts

Quantification of CPT in extracts, prepared from mycelia and broth was done on the basis of calibration curves established by injecting six concentrations of the

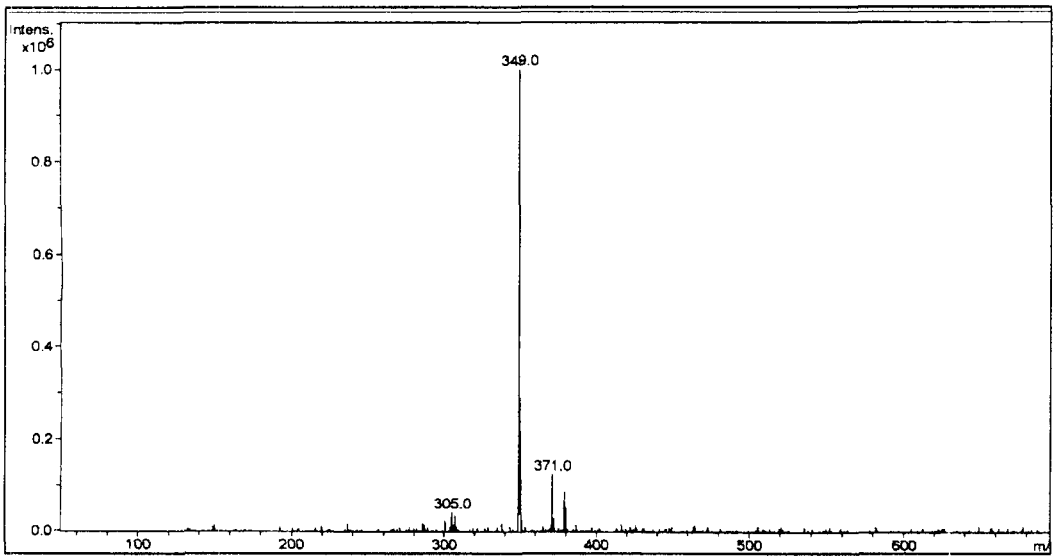


Fig.24: ES-MS spectra of CPT

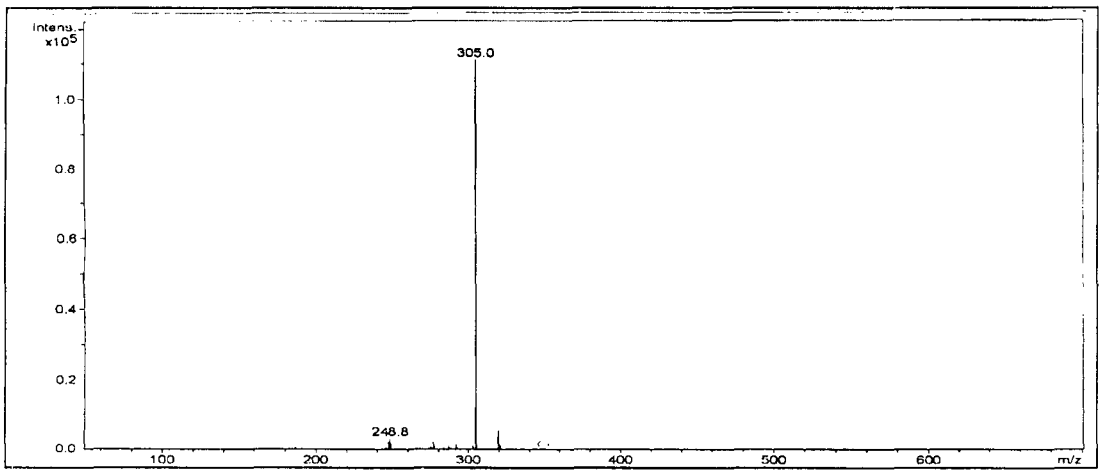


Fig.25: ES-MS/MS spectra of CPT at m/z 305

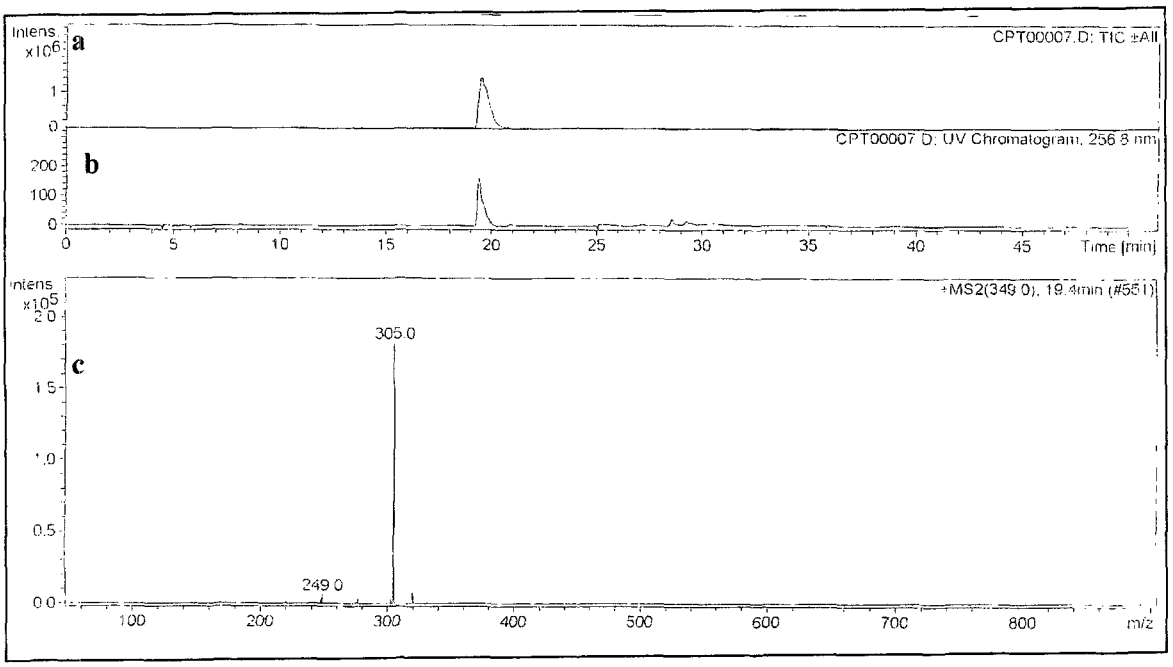


Fig. 26: Total Ion Current Trace (a) from SRM, UV- (DAD) chromatogram (b)at 256 nm and LC-ES- MS/MS (c) of CPT

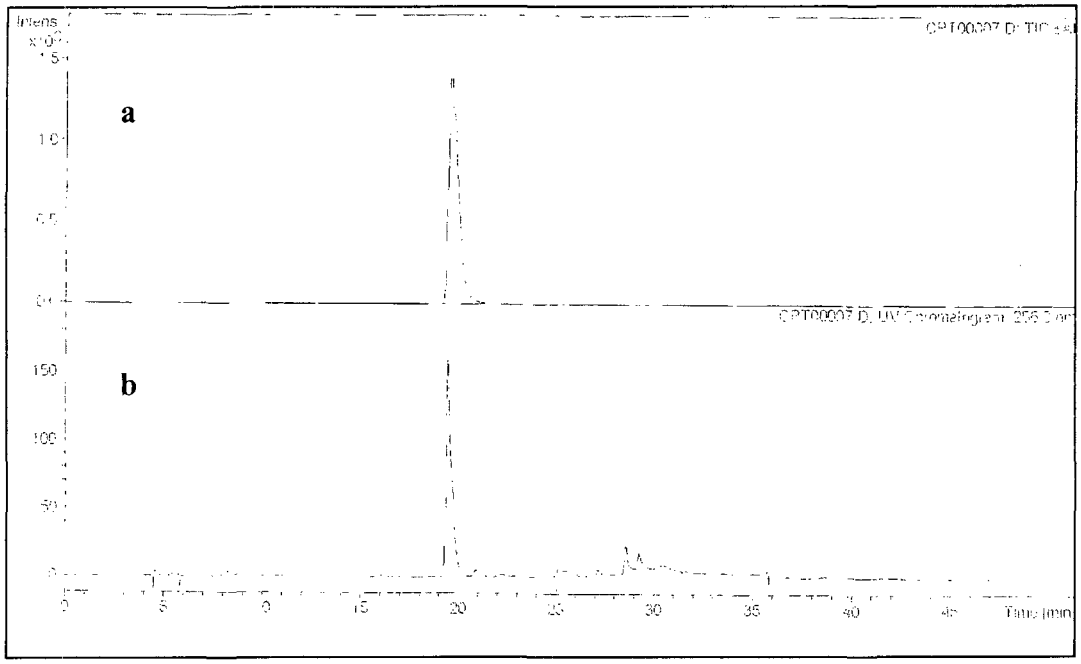


Fig. 27: Total Ion Current (a) trace from SRM, and UV-(DAD) Chromatogram (b) of CPT

CPT standard in the range of 5 ng to 50 ng every time before the sample analysis. Quantification of CPT was carried out using the SRM detection of the molecule at above-mentioned concentrations. Linear calibration curves for the CPT within the concentration range of 5 ng to 50 ng (R^2=curve coefficient 0.9998695) were obtained. Linearity of the standard curve showed consistent reproducibility till concentration of 100 ng of the standard, though a reduced R^2 = 0.9936426 was observed. Since the concentration of CPT in the samples was lying with in the range of 5 ng to 50 ng, standard curve with this concentration range was used. Successive lower concentrations of CPT standard were injected and limit of detection was estimated to be 20 pg. To study reproducibility of the LC-MS/MS method and to access run-to-run precision, six replicate analysis of 0.5 mgl^{-1} standard solution of CPT were carried out under optimal LC-MS conditions. The relative deviation was found to be 0.4%.

Separation of 9-Methoxycamptothecin and Camptothecin from *Nothapodytes foetida* by semi-preparative HPLC

Chromatographic conditions

Analytical HPLC instrumentation

Analytical HPLC was performed on a Gilson (Villiers Le Bel, France) HPLC with 305 pump and 10SC pump head, 306 manometric module, 115 UV detector set at 256 nm, Rheodyne (Cotati, CA) injector 7725i with 50-µl sample loop was used with Merck (Darmstadt, Germany) column Lichrosphere RP-18 (5-µm particle size, 4x100-mm size) was used to separate the compounds.

Semi-preparative HPLC instrumentation

A Gilson semi-preparative HPLC system consisting of "305" pump and "25SC" pump head, "306" manometric module, "7725i" Rheodyne injector with 300µl sample loop, 116 UV detector set at 272 nm and Rainin Dynamax semiprep C-18

column (100 × 5 mm, 2 μm particle size) was used along with Gilson 201 fraction collector.

Liquid chromatography-mass spectrometry

Liquid chromatography (LC)-mass spectrometry (MS) experiments were performed on a Bruker (Bremen, Germany) Esquire 3000 ion trap mass spectrometer with an ESI-interface and connected to an Agilent (1100 series) binary pump, photodiode array detector, automatic sample injection module and thermostatic column oven (Agilent, Palo Aito, CA).

Isolation of 9-methoxycamptothecin and camptothecin and processing of the collected fractions prepared from plant material

Plant material was collected from RRL botanical garden, cultivated from the seeds obtained from the Mahabaleshwar forests, India. A finely powdered aerial portion of plant material (100 g) was extracted with methanol in a Soxhlet apparatus. The extract was filtered and concentrated on a rotatory evaporator. The crude extract (9.5 g) was suspended in 100 ml of water, which was further partitioned with petroleum ether (3 × 50 ml), and chloroform (3 × 50 ml) successively. The chloroform extract was dried over anhydrous sodium sulphate, concentrated on a rota-evaporator and was dissolved in boiling chloroform: methanol (80:20 v/v). The clear solution on standing for 4-6 hrs at 5° C, camptothecin precipitates out as a yellow fine powder.

Mother liquor after removing the solvent (10 mg) was dissolved in 10 ml of chloroform: methanol mixture (80:20 v/v) and subjected to an analytical HPLC system using UV detector set at 256 nm (Fig. 28). The Mobile phase consisting of premixed water: acetonitrile (25:75) [filtered, degassed on Millex HV filter (0.45 Millipore)] injected and eluted at a flow rate of 1 ml/min. The chromatogram of the filtered extract was plotted on "HP 3395" integrator. The peaks of camptothecin and 9-methoxycamptothecin were detected at retention time (t_R) of 13.079 min and 22.13 min respectively and confirmed by co-spiking with their corresponding standards.

Semi-preparative HPLC of the extract

The mother liquor of the extract (432 mg) was subjected to semi preparative HPLC. A premixed solvent system consisting of water: acetronitrile (25:75) was isocratically pumped at a flow rate of 3 ml min^{-1}. 300 µl of the extract was loaded on the Rheodyne injector. Two peaks were collected in clean pre-weighed flasks. Camptothecin (I) was collected from 14.5 to 16 min and 9-methoxy-camptothecin (II) was collected from 23 to 25 min (Fig. 29). After 20, such collections the pooled eluates in an Erlenmeyer's flask were visualized under UV showing blue fluorescence in fraction (I) and yellow in fraction (II). The azeotrope phase was removed by rotatory evaporation under reduced pressure. Removal of the organic phase from the respective pooled fractions yielded camptothecin (15 mg) and 9-methoxy-camptothecin (42 mg) respectively.

Purity check of the collected fractions

To check the purity, the residue was dissolved in chloroform: methanol (80:20 v/v). The prepared solution was analyzed on HP-100 HPLC system (Agilent) linked to Bruker Daltonics Esquire 3000 mass spectrometer with an ESI source (LC-ESI-MS-MS) scanned over a mass range between *m/z* 100 to 900. 10 µl of fraction (I) and (II) solutions were injected separately with an autoinjector on a Merck Chromolith C_{18} column (100 × 4 mm) and eluted with water: acetonitrile (25:75) at a flow rate of 0.8 ml min^{-1}. The fraction (I) eluting at t_R of 13.08 min, exhibited molecular adduct $(M+H)^+$ at *m/z.* 349.1 (Fig. 30). The fraction (II) eluting at t_R of 22.14 min exhibited molecular adduct $(M+H)^+$ at *m/z* 379.1.

Isolation and characterization of a Furan derivative

Fungal culture was grown and harvested in the same manner as described in Chapter 3.Besides extracting CPT from fungal culture, the residual pellets were exhaustively extracted (Fig. 14) with equal volume of n-butanol (70% v/v) four times (1l x 4). Concentration of n-butanol extract on rotary evaporator under reduced pressure gave brown oily residue. The butanol extract of residual pellets

was subjected to HPLC and showed prominent peak on HPLC (Fig. 31). To isolate the major component in the crude extract, the residue (4.5 g) was subjected to the chromatography over silica gel column (1000 g, 60-120 mesh) eluting with chloroform – methanol gradient (90:10,2 l; 80:20,2 l; 50:50,2 l; 100:0,1 l) to give five fractions. (F-1-5). F-1, F-2 andF-3 showed the same pattern on TLC and were pooled. The pooled fraction (1.1g) was rechromatographed further over silica gel (50 g, 60-120 mesh) using chloroform (100%) to yield pure compound (30 mg).

HPLC Analysis

HPLC separation was performed using Luna RP-18 column (2mm i.d., length 150 mm, particle size 3 μm) and a safety guard column (Phenomenex, Torrance, CA, USA) at 30 °C. The mobile phase was water (A) and methanol (B) run in the following manner (0.01 min 90 % A and 10 % B, 40.0 min 100 % B, 45 min 100% B, 55 min 90% A and 10% B and 60 min 90 % A and 10% B v/v) at a flow rate of 200 μl min^{-1} detection: UV at λ_{max} = 283 nm, t_R =10.98 min. 10 μl of sample was injected in $CHCl_3$:MeOH (9:1).

Isolation and characterization of Ergosterol from endophyte

Spore suspension (seed) harvested from Roux bottles as described in Chapter 3 in present study was inoculated as seed into 40 flasks (500 ml) each containing 100 g of wheat bran supplemented with Sabouraud broth. Culture temperature, initial moisture content and cultivation time was 28 ± 2 °C, 80% and 28 days respectively. Moreover four flasks without inoculum were prepared as above for comparison to prove all compounds to be the endophytic fungal metabolites by TLC and LC-MS examinations.

The crude solid fermentation product (1.5 kg not completely dried) collected after desiccation and grinding of 40 flasks of the cultures was exhaustively extracted with $CHCl_3$: MeOH (4:1, v/v) mixture (15 l x 3). Evaporation of the solvent in vacuo yielded a semisolid black residue (17 g not totally dried).

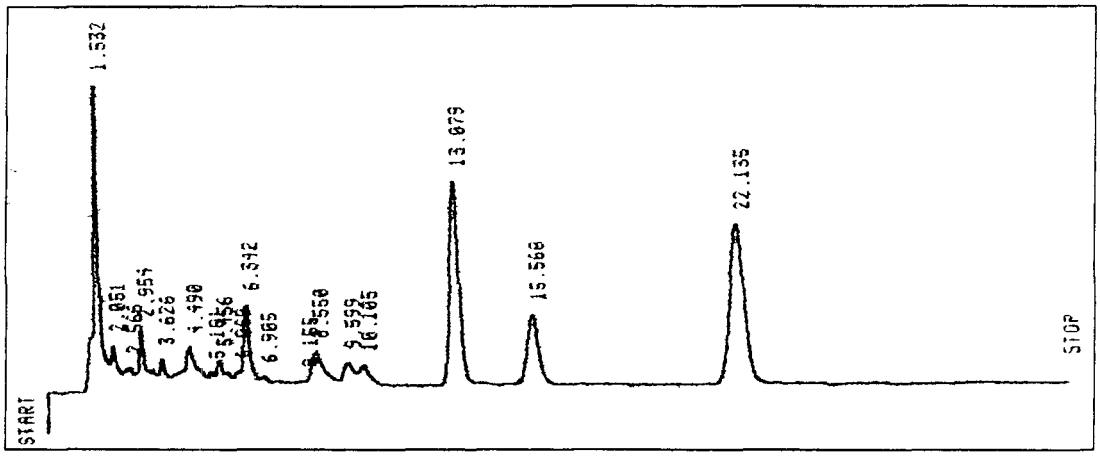


Fig. 28: HPLC Chromatogram of the crude extract of *N. foetida.* t_R camptothecin (13.079) minutes and 9-methoxy-camptothcin (22.135) minutes.

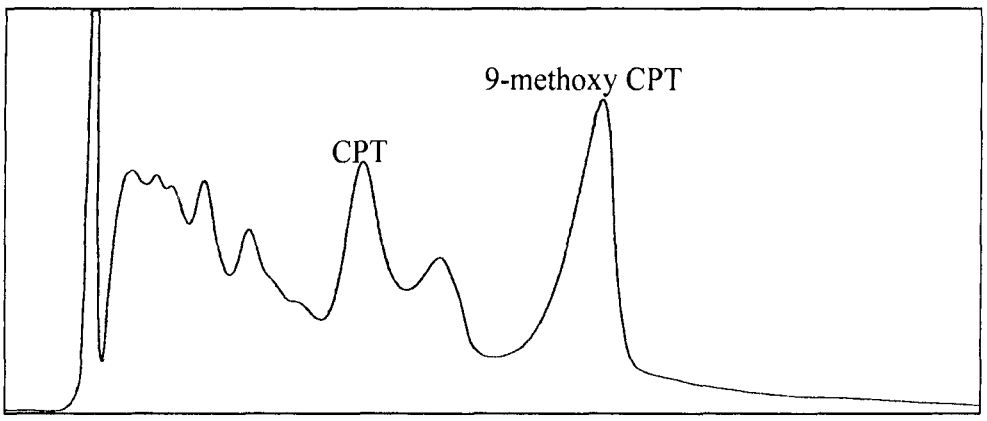


Fig. 29: Semi-preparative HPLC profile of crude extract of *N. foetida*. Each division on the x-axis depicts time interval of two minutes.

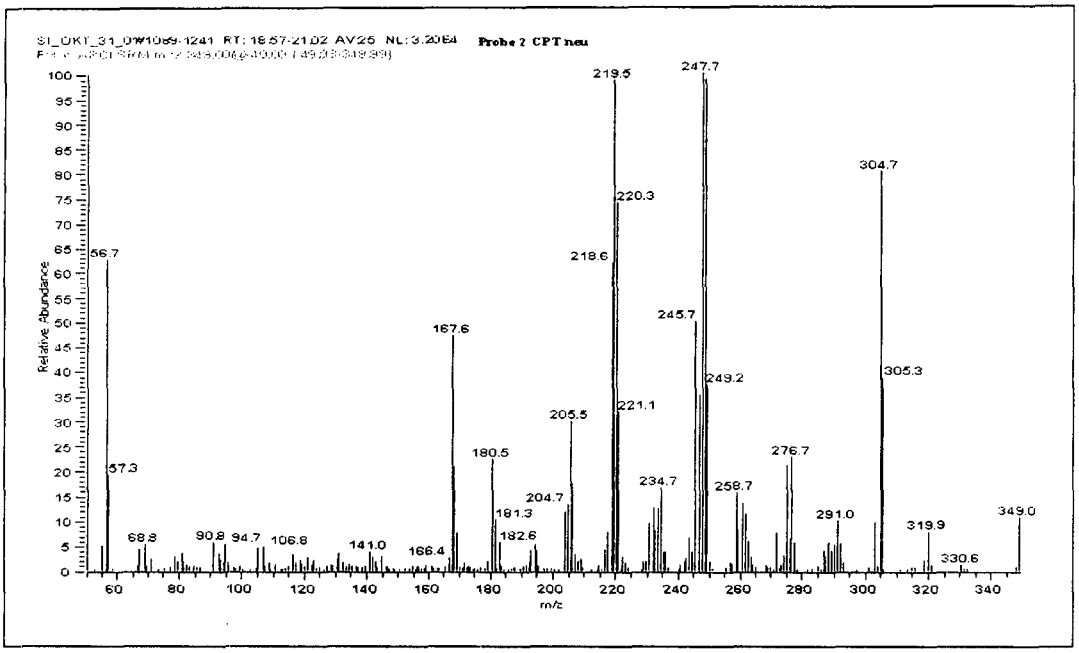


Fig. 30: Mass spectrum of camptothecin

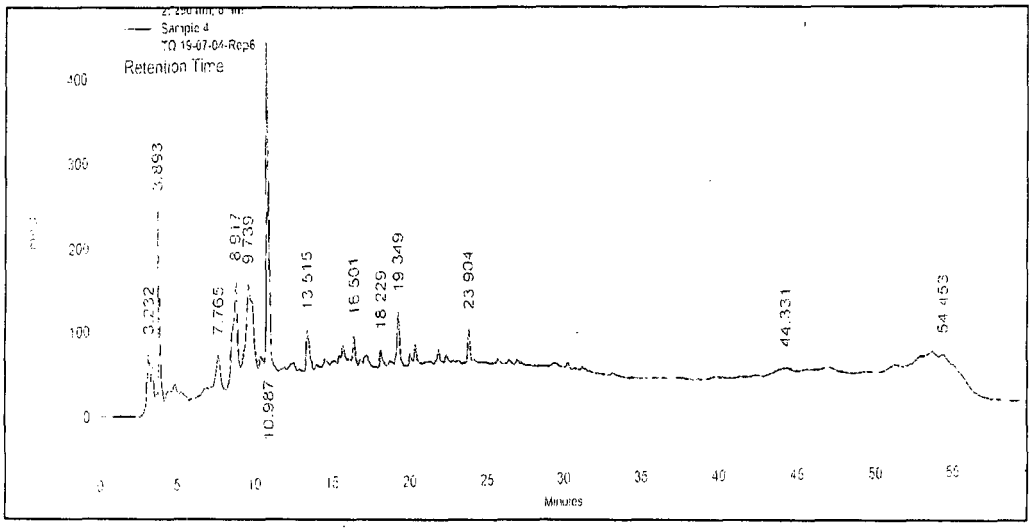


Fig. 31: HPLC profile of n-butanolic extract of RJMEF001 isolate

Column chromatography

14.1 g crude extract of mycelia was subjected to chromatography over silica gel column (1200g, 60-120 mesh) eluting with benzene-ethyl acetate gradient (90:10, 2 l; 80:20,2 l; 50:50,2 l; 100:0,1l) to give 8 fractions. These fractions were shown to be of no interest by TLC. Further elution was carried out using ethyl acetate-methanol mixture of growing polarity (90:10,2 l; 80:20,2 l; 50:50,2 l; 100:0,1 l) to give four fractions. The fractions eluted in ethyl acetate- methanol mixture (90:10, 3.4 g) showed same pattern on TLC and were pooled. Pooled fraction was rechromatographed on silica gel column (280 g, 60-120 mesh) eluting with chloroform- methanol mixtures (80:20-90:10, each 1 l) to afford pure compound. The compound was identified on the basis of spectral data as ergosterol.

The sterile medium was extracted following exactly the procedure as with that of the fungal culture. LC-MS comparisons demonstrated that the medium did not contain any of the fungal metabolites.

Results

Identification and characterization of CPT from fungus

The identification of CPT (Fig. 1 a) in fungal culture was confirmed by super-imposed UV, IR spectra, optical rotation, CD/ORD, LC/MS/MS, ^{1}H NMR and ^{13}C-NMR (Table 8). The electron impact mass spectrum (EI-MS) of fungal camptothecin showed molecular ion peak *m/z* 348 with characteristic fragment at *m/z* 319 (M-ethyl), *m/z* 304 (M-CO_2), *m/z* 291(*m/z* 319-CO), *m/z* 290 (*m/z* 319-CHO), *m/z* 275(*m/z* 304-ethyl), *m/z* 248 (*m/z* 275-HCN), *m/z* 247 (*m/z* 275- CO) and *m/z* 219 (*m/z* 247-CO) which clearly was in tune with the reported spectrum of the standard camptothecin (Hengel *et al.*, 1992). In ESI-MS, the molecular ion of the camptothecin actually exhibits m/z 349 $(M+H)^{+}$ mode characteristics fragment peaks were observed (Fig. 32a,b) and the fragmentation pattern confirmed the identity of camptothecin.

Quantitation of camptothecin in mycelial and broth extracts

Organic solvent extracts of mycelia and broth of surface culture were found to contain CPT after 14 days. The maximum yield of CPT per 100 g dry weight of mycelia was found to be 4.28 ± 0.05 (the results are mean of six experiments) mg/100 g mycelium (dry weight) and 250 ± 20 μgl^{-1} of broth. No CPT was detected in uninoculated as well as in inoculated culture broth at the beginning of the incubation (zero days). The experiment was carried out six times in replicates of three along with equal number of standard samples to assess the repeatability and reproducibility of the results.

An LC/ESI/MS/MS assay for the quantification of CPT in mycelia and fermentation broth of a fungal culture isolated from *N. foetida* has been developed and validated. The method is linear over a wide concentration range with a correlation co-efficient greater than 0.996. The analytical protocol based on LC/MS has successfully been employed in present study for the analysis of camptothecin in crude extracts obtained from plant sources, fermentation broths and mycelia cultured in the laboratory. The assay method is sensitive and selective, allows minimal matrix interferences and requires a very simple sample clean-up

procedure. The results obtained during the fermentation experiments for the production of camptothecin demonstrate the usefulness of the assay in monitoring the metabolic flux of CPT in endophytes residing in the plants.

Isolation of camptothecin standard from plant material

The purity of collected fractions was determined on the basis of LC run separately. Camptothecin and 9-methoxy-camptothecin eluted at t_R of 13.08 and 22.14 min respectively, were confirmed simultaneously by comparison of total ion chromatogram (TIC) with a LC-UV chromatogram. Complete overlap of the TIC and UV chromatograms pointed out the purity of the collected compounds.

From the preparative HPLC a number of fractions were collected. Fraction (I) camptothecin (95% pure) was crystallized from chloroform: methanol (80:20 v/v) to give light yellow crystals m.p. 273-274°C, $C_{20}H_{16}N_2O_4$; $[M+H]^+$ 349.1 (cal. CHN 348.11), $[\alpha]_D^{25} = +34.8$ (*c* 0.40, 8:2 $CHCl_3$/MeOH; lit. +35°). Analytical and spectral data were in agreement with those reported in literature (Wani *et al.*, 1966; Long and Cordell, 1990). Fraction (II) on crystallization from chloroform: methanol (80:20 v/v) gave yellow crystals. m.p. 266-269° C, $C_{21}H_{18}N_2O_5$; M^+ 378 (cal. CHN 378.38) $[\alpha]_D^{25} = -98.54$ (*c* 0.29py). TLC, UV, HPLC and LC/MS data (Fig. 33) confirmed its identity and purity (95%) as 9-methoxy camptothecin (Fig. 34).

Identification and characterization of 5-(hydroxymethyl)-2-furfuraldehyde from fungus–RJMEF001

On repeated Column chromatography of the n-butanol extract of residual pellets of endophytic fungus on silica gel; the fractions which showed identical single spot on TLC [Rf 0.55; $CHCl_3$: MeOH; 9:1] were pooled. After removing the solvent, attempts were made to crystallize the pure sample in different solvents and their mixtures but no success could be achieved to get a crystalline mass. The semisolid material was dried in a drying piston and subjected to microanalysis for its elements, where it analyzed for $C_6H_6O_3$ [Found C= 56.91%; H = 4.97%; 0 = 37.89%; while $C_6H_6O_3$ analysed for C= 57.14%; H= 4.80% and 0= 38.06%]. The pure sample which has a pleasant smell was subjected to spectral analysis.^{1}HNMR

Table 8: Comparison of spectral data of camptothecin isolated from plant and fungus

Spectra	Plant- CPT	Fungal-CPT
1.Uvλ_{max}.[nm] (intens.) ($CHCl_3$ / MeOH 4:1)	256 (0.820), 290 (0.176),361 (0.557), 371 (sh, 0.536) ε_{361} = 19.350	256 (0.800), 290 (0.167), 361 (0.527), 371 (sh, .511) ε_{361} = 18.300
2.IR (KBr) [cm^{-1}]ν_{max}	1751, 1741, 1652, 1601, 1580, 1500, 1438, 1345, 1323, 1252, 1234,1157, 1156	1751, 1740, 1652,1602, 1580, 1500,1438, 1349, 1324,1252, 1234, 1197, 1157
3.Optical rotation $[\alpha]^{30}_D$ ($CHCl_3$ / MeOH 4:1)	+31.3°	+29.6°
4.CD ($CHCl_3$ / MeOH 4:1) max ($\Delta\varepsilon$)	201 (-4), 211 (5), 216 (9), 221(-7), 229 (sh, 0), 238(33), 251(sh, 5) 257 (-2), 263 (8).	295 (4) 214 (-6), 220, 219 (2), 237 (36), 257 (sh,10).
5. Mass spectrometry 5.1 High resolution FAB-MS	349.1176 $[M+H]^+$ $C_{20}H_{17}N_2O_4$, error 1.2 mmu	349.1147$[M+H]^+$ $C_{20}H_{17}N_2O_4$, error 1.1mmu
5.2 High resolution ESI-IRMPD-MS/MS	m/z 349.11920 m/z 305.12928 m/z 277.13424 m/z 249.10283	m/z 349.11925 m/z 305.12930 m/z 277.13429 m/z 249.10288
6. ^{1}H NMR (200MHz,DMSO)	δ 8.70 (s,1H), 8.16 (m,2H), 7.88(t,1H, J =7.02,Hz), 7.73(t,1H, J =7.64,Hz), 7.37(S,1H), 6.57(s,1H), 5.44(S,2H), 5.30(s,2H), 1.86(m,2H), 0.89(t,3H,J=7.4Hz),	Identical with plant CPT
7. ^{13}C-NMR (151 MH_z, DMSO)	δ = 152.5 (C2), 145.4 (C3), 50.2 (C5), 129.8, (C6), 131.5 (C7), 128.5 (C8), 127.6 (C9), 127.9 (C10), 130.3 (C11), 129.0 (C12), 147.9 (C13), 96.7 (C14), 149.9 (C15), 119.0 (C16), 156.8 (C16a), 65.2 (C17), 7,7 (C18), 30.3 (C19), 72.3 (C20)	Identical with plant CPT ± 0.05 ppm shift

(1) **UV-Visible** -Instrument: Varian CARY 100 BIO (2)**FTIR**-Instrument: Bruker IFS (KBr) (3) **Optical rotation**-Instrument: Perkin Elmer 341 Polarimeter (4) **CD/ORD**-Instrument: JASCO J715 spectropolarimeter, Hellma Precision quartz glass suprasil cuvette. (5)**Mass spectrometry**- Instrument: JEOL JMS/SX 102 A (6) **^{1}H NMR- ^{13}C-NMR** -Instument: Bruker AMX 600, solvent DMSO

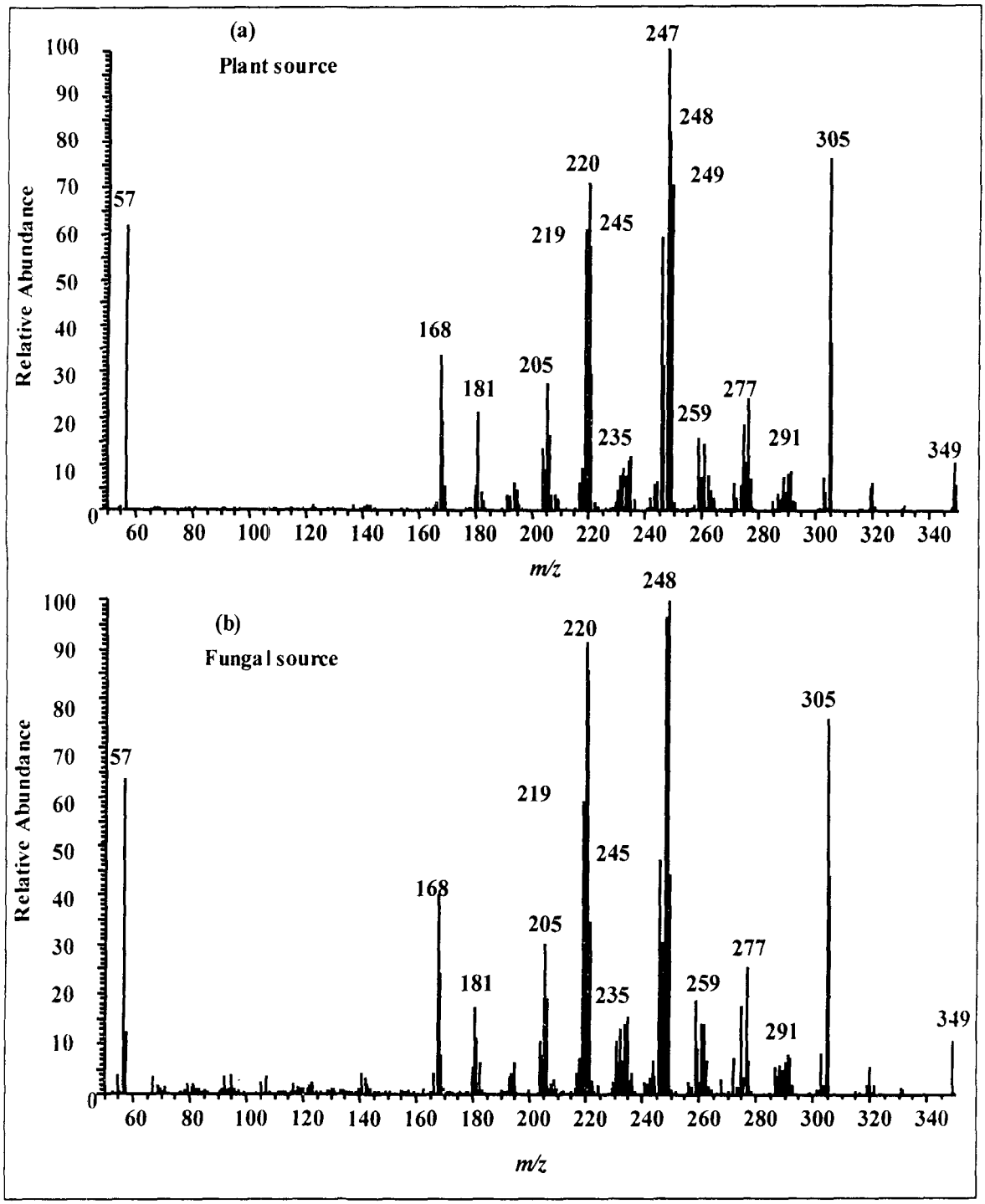
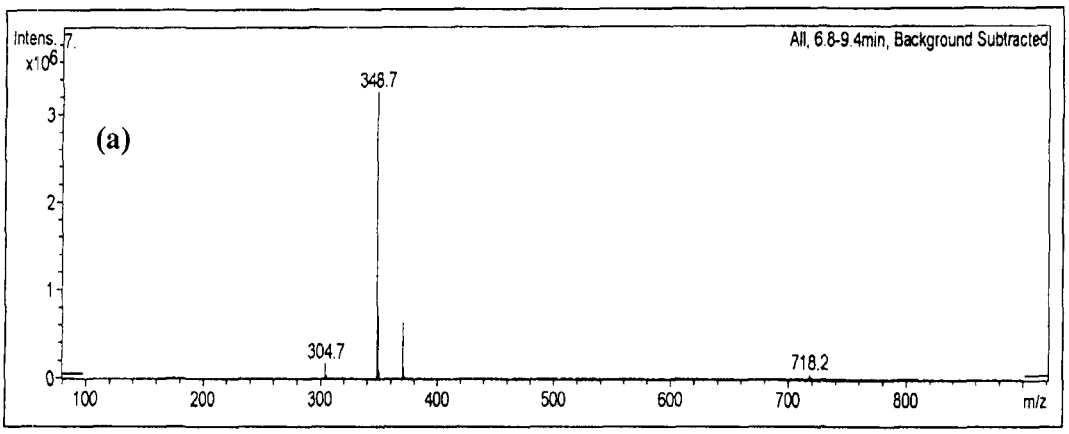


Fig. 32: ESI-MS/MS spectrum of CPT from: (a) Plant source (b) Fungal source



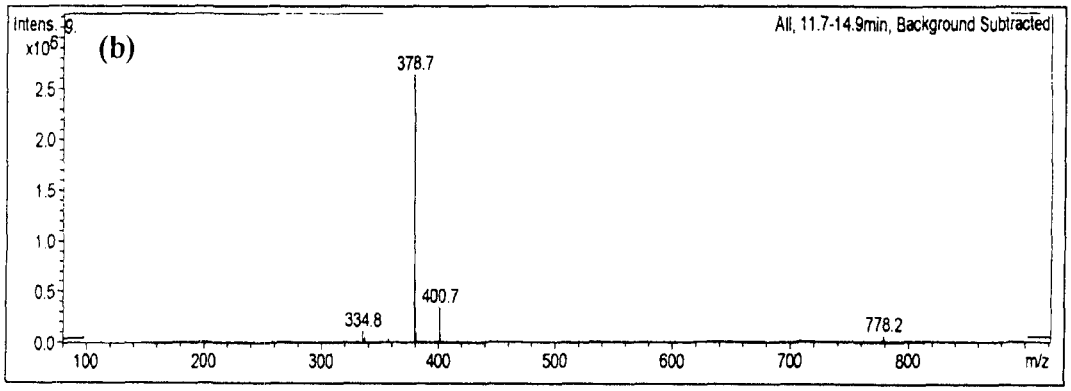


Fig. 33: Mass spectra and fragmentation of (a) Camptothecin & (b) 9-methoxy camptothecin

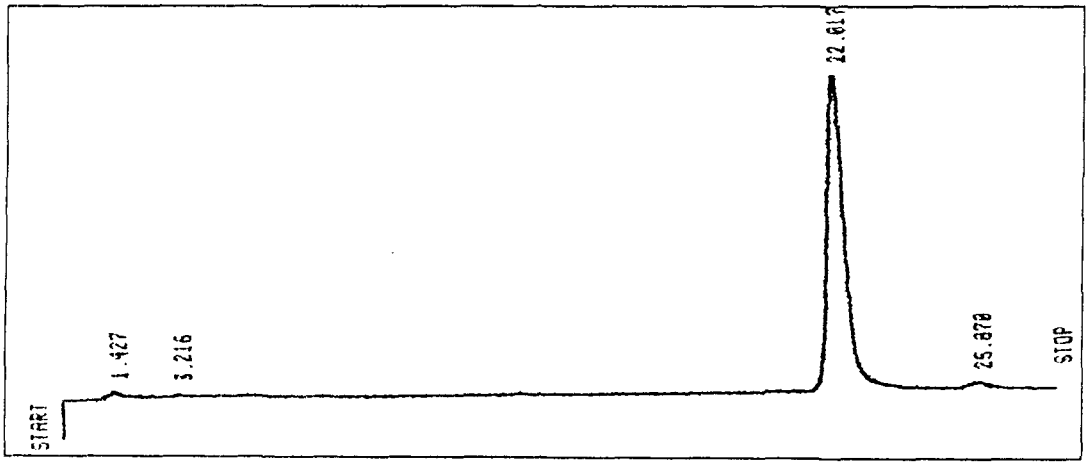


Fig. 34: HPLC profile of isolated 9-methoxy camptothecin.

(Bruker-500MHz, $CDCl_3$; δ values, ref. to TMS as 0 ppm) showed a broad singlet at 4.66 for two protons; two doublets at 6.42 and 7.13 with J=3.4 Hz each integrating for one proton; indicating the possibility of a furan ring. A down field singlet at 9.55 integrating for one proton appeared to be on aldehydic proton attached to a furan ring. Though ^1H accounted for all the six protons; it was difficult to assign the position of a two-proton singlet at 4.66. So detailed ^{13}C NMR studies were undertaken.^{13}C spectra ($CDCl_3$, δ ppm, TMS=0) showed signals for all the six carbons. Signal at 57.71 indicated it to be a shielded OCH_2 by DEPT studies (at 135^0 pulse angles and corresponded to ^1H signal at 4.66 as shown by two Dimensional heteronuclear cozy studies, similarly two furan proton containing carbons were seen at 109.93 and 122.36 corresponding to ^1H protons at 6.42 and 7.13 protons respectively as indicated by 2D Heterocosy studies. Aldehydic carbon was assigned to singlet of carbon at 177.57 corresponding to ^1H NMR signal at 9.55. Quaternary carbons were clearly visible at 152.51 and 160.13. Down field shift of proton and carbon at C-3 compared with C-4 is due to the fact that it is β to α-β unsaturated carbonyl at C-2. Thus the compound appeared to be 5-(hydroxymthyl)-2 furfuraldehyde. The proper assignments are given in Fig.35.

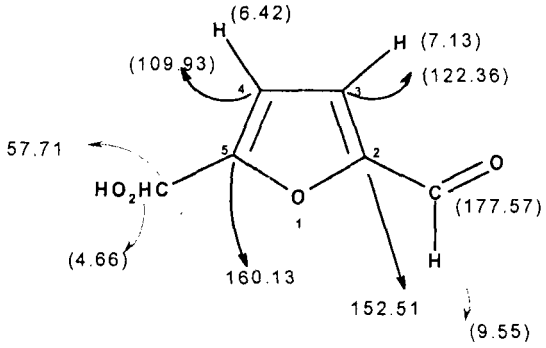


Fig. 35: 5-(hydroxymethyl)-2-furfuraldehyde

MS was studied on GCMS (QP2000 Shimadzu, Japan) where it showed M^+ at 126.11 with important fragments at m/z 101, 97, 96 and at 67 confirming the compound to be 5-(hydroxymethyl)-2-furfuraldehyde). Infra red studies showed characteristic peak at 1691 cm^{-1} for aldehydic carbonyl and showed in UV λ_{max}^{EtOH} at 283 nm.

This compound has been reported to be an important product when fructose is subjected to fermentation. Though conversion of fructose to 5-(hydroxymethyl)-2-furaldeyde is reported) but occurrence of this product from glucose appears to be rare but not surprising. It is well known that glucose changes to fructose under acidic conditions involving transfer of hydrogen from C-2 of D-glucose to C-1 of D-fructose (Harris and Featter, 1972). Such hydride transfer reactions are thus possible under the conditions, which mimic the acid catalyzed reactions such as dehydration for the conversion of pentoses to 2-furaldehyde.

Identification and characterization of Ergosterol from fungus

On repeated column chromatography, fractions which showed identical TLC pattern with single spot (Rf =0.47; Solvents; $CHCl_3$: MeOH; 9:1), were pooled and after removal of solvent, the residue was crystallized from diethyl ether, m.p 168°. It was subjected to element analysis where it correctly analyzed for $C_{28}H_{44}O$, showed M^+ at 393.63 and responded to Liebermann & Bunchard; test. The compound easily formed a monoacetate on treatment with acetic anhydride and pyridine, which analysed for $C_{30}H_{46}O_2$; m.p. 179°.

Detailed ^{1}HNMR coupled with ^{13}C NMR studies were made on 500 MHz for ^{1}H and the results are discussed as follows: ^{1}HNMR was run in $CDCl_3$ (δ, values ppm with reference to TMS=0) where it showed a singlet (3H) at 0.63, another singlets at 0.946 (3H), three doublet at 0.828, 0.84, 1.04 each integrating for three protons, a multiplet at 3.67 a miltiplet centered at 5.16 (2H); two doublets at 5.37 and 5.56 ^{1}H (with J= 5.4 Hz in both) and appear to be mutually coupling with dihedral angle close to zero. The data fits well with ergosterol $C_{28}H_{44}O$, m.p 179° (Fig. 36). The ^{13}C values were taken on the same instrument in $CDCl_3$. Proper assignments were taken by DEPT at 45, 135° studies. The results are tabulated below (Table 9):

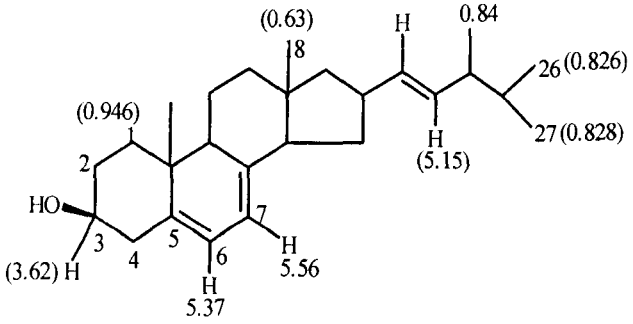


Fig. 36: Ergosterol

Table 9: ^{13}C values of ergosterol

1. 38.41	**2.** 32.02	**3.** 70.48	**4.** 40.82
5. 141.38	**6.** 119.60	**7.** 116.31	**8.** 139.82
9. 46.27	**10.** 37.05	**11.** 21.12	**12.** 39.11
13. 42.84	**14.** 54.58	**15.** 23.01	**16.** 28.30
17. 55.76	**18.** 12.06	**19.** 16.33	**20.** 40.43
21. 19.66	**22.** 132.09	**23.** 135.59	**24.** 42.84
25. 33.11	**26.** 19.96	**27.** 21.12	**28.** 17.62

Isolation and characterization of new molecules of endophytic fungus

The CPT free ethyl acetate fraction of the fungus could not be subjected to classical chromatographic techniques for the separation and purification of the constituents due to paucity of the material. However, after a number of trial runs, it was possible to subject the extract to LC-NMR (^{1}H) and LC/MS coupled with further MS-MS instrumental techniques to get an insight into the structure of molecules, which are the constituents of the ethyl acetate extract.

LC-NMR was run on column RP-18 on a reverse phase mode using Acetonitrile: D_2O in different proportions (0-0.01 min. 90:10, 0.01-5.00 min 90:10, 5-20 min 40:60, 20-25 min 20:98, 25-30 min 2:98, 30-35 min 90:10, 35-40 min 90:10) at the flow rate of 0.6 ml/min and detected at UV λ 254 nm and showed peaks at retention time as 3.72, 15.36, 19.11 and 20.94. Details of which are given in the enclosed copy of the graph (Fig. 37 a, b).

Peak No 3 showed characteristic^{1}H-NMR after usual solvent suppression was made. Two prominent singlets each integrating for 3 protons at 3.78 and 3.72 could be assigned to two methoxyls attached to the aromatic ring. Two classical AB doublets at δ 6.25 and 7.23 with J=15.5 Hz could easily be assigned to α and β proton of an α-β unsaturated carbonyl system. The NMR graph also showed two doublets at 7.12 and 6.8 with coupling constant (J) of 8.2 Hz along with a singlet at 7.13 (slightly meta coupled), which clearly indicated a substituted cinnamic acid moiety. Since ^{1}H NMR also showed two doublets at 6.64 and 6.68 (J=8.2) and a singlet at 6.8 made it possible to consider the molecules as a phenoxy substituted ferulic or caffeic acid. The possibility of an ester of a substituted phenol was ruled out because of the molecule being extremely polar. This was further supported by LC/MS, which showed M^+ at 316 with fairly large fragment at *m/z* 299 (M-17) which is possible if it is assumed that the substituted cinnamic acid is in free carboxylic acid form. Possibility of an amide which can also show M-17 fragment is also ruled out due to high polarity of the molecule.

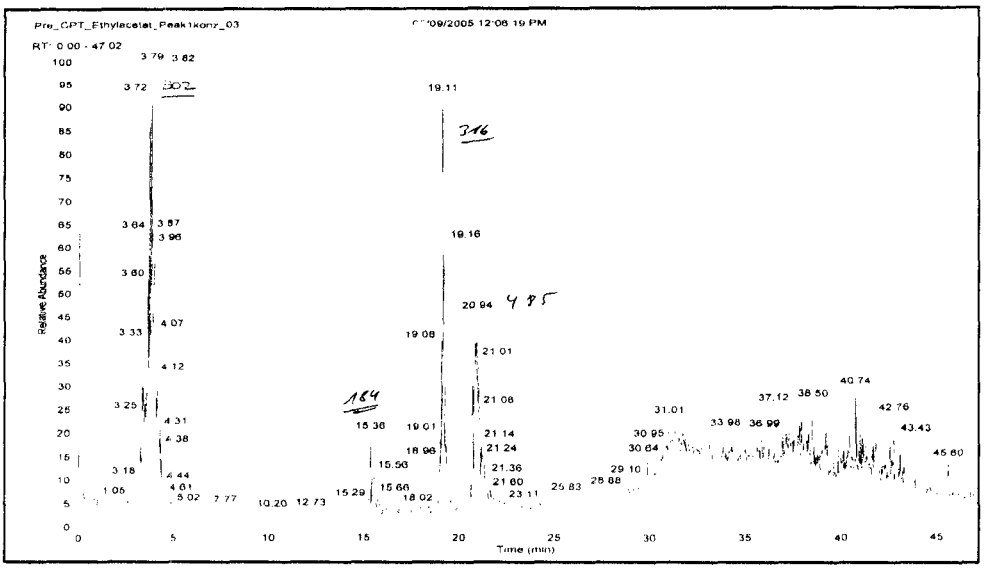


Fig. 37: (a) LC Profile of ethyl acetate fraction

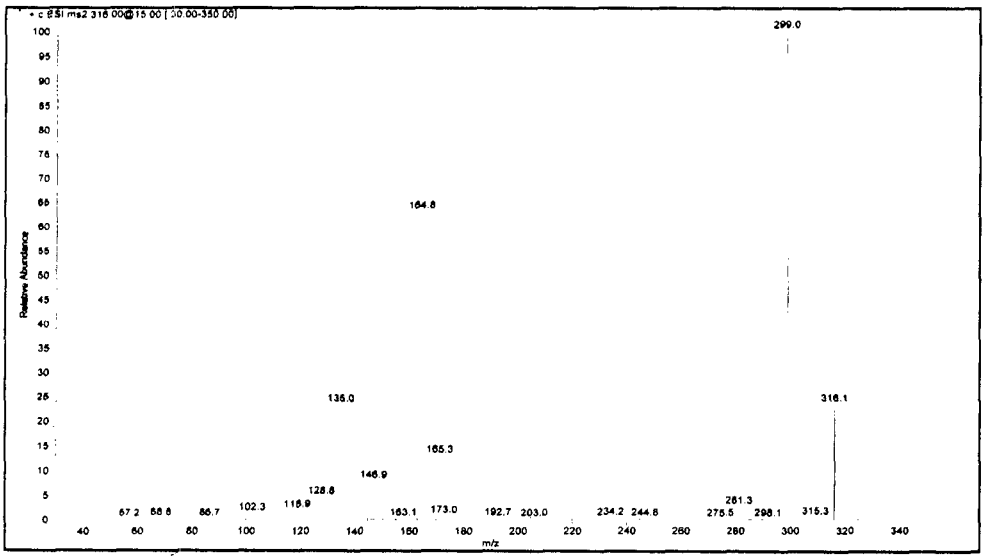


Fig. 37: (b) MS Profile of ethyl acetate fraction

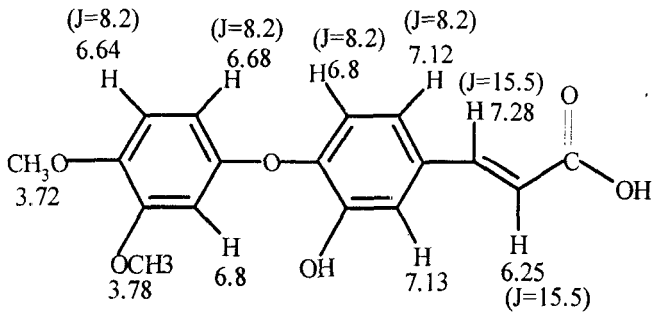


Fig. 38: 4-(3, 4-dimethoxy-phenyl-1-oxy)-caffeic acid

LC/MS for a peak which corresponds to peak 3 of LC/NMR besides showing M^+ 316 showed other fragments when MS/MS mode was considered. Fragment at *m/z* 299, *m/z* 164 and *m/z* 135 indicated in support to ^1H NMR that the molecule is a caffeic acid derivative rather than ferulic acid derivative as shown by the following fragmentation pattern:

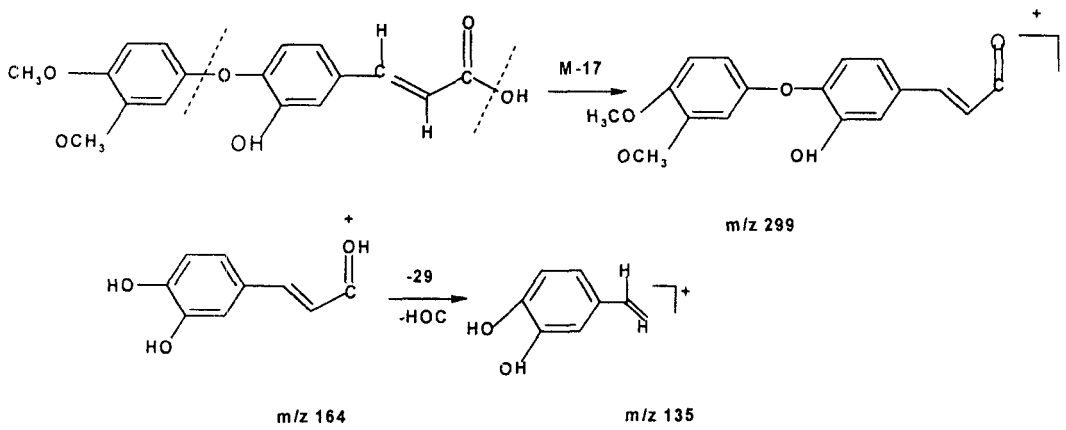
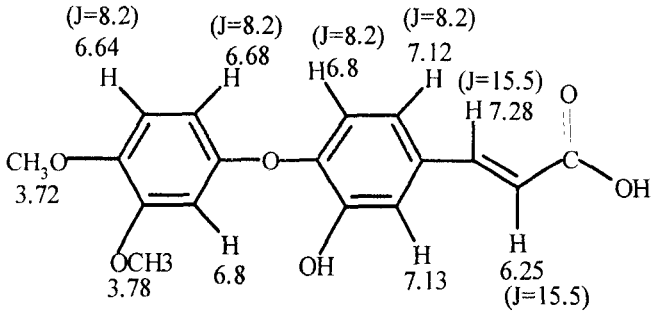


Fig. 39: MS fragmentation

Therefore the compound is tentatively identified as 4-(3,4-dimethoxyl –phenyl-1-oxy)-caffeic acid, which appears to be new to the literature.

Peak 2

Peak 2 which did not appear as a sharp peak in LC/NMR but appeared as a well defined peak in LC/MS where it showed M^+ 302, while its corresponding peak in LC/ NMR showed ^1H NMR very close to peak 3 NMR except for the missing one methoxyl. However, from MS-2 it was clear to be similar in structure to the



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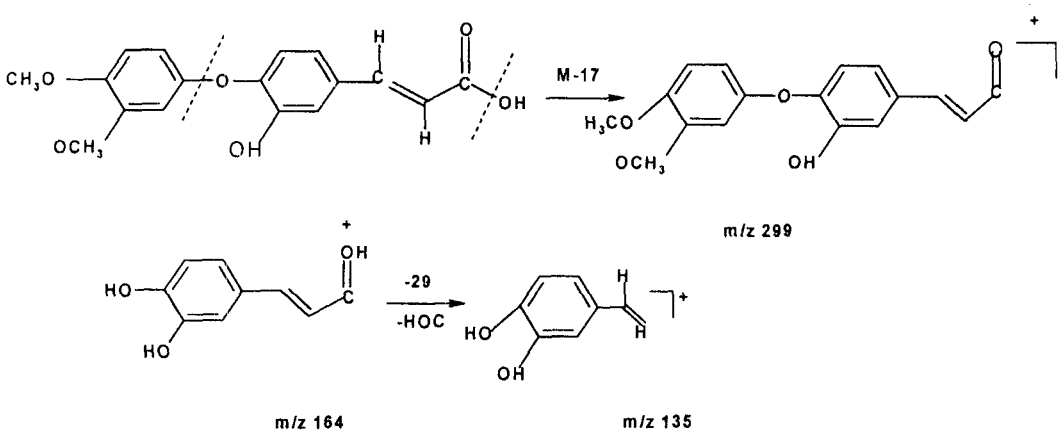

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compound corresponding to peak-3. Tentatively its structure can be assigned as follows:

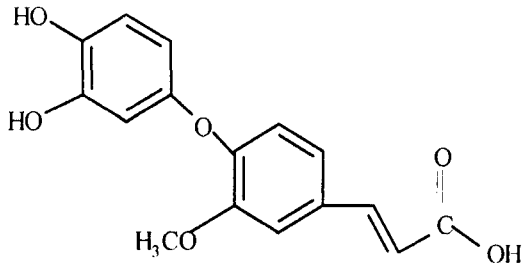


Fig. 40: 4-(3,4-dihydroxy phenyl-1-oxy)-ferulic acid

The compound therefore might be a ferulic acid derivative rather than coffeic acid derivative as in the case with compound corresponding to peak 3 and is identified as 4-(3,4-dihydroxy phenyl-1-oxy)-ferulic acid and also appears to be new to the literature.

Peak 5

The compound corresponding to peak No 5 appeared to be more complex than the other two as already described. It should eight doublets rather than six as the case was with compound corresponding to peak No 3. Since it showed only two methoxyl and typical two doublets at 6.30 (J=16Hz) and at 7.16 (J=16Hz) which appear to be in tune with ferulic acid. M^+ at 484 with important fragments in MS-2 at *m/z* 302 and *m/z* 184. From ^1H NMR and MS-2, the compound appeared to be a peroxy ester of (corresponding to peak No 2) a poly-substituted salicylic acid, which was detected in LC/MS with M^+ 184. Therefore tentative structure can be assigned as follows:

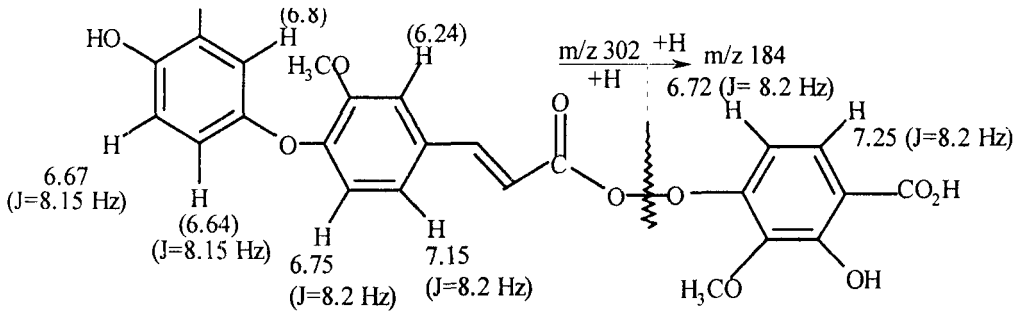


Fig. 41: 4[4-(3,4-dihydroxyphenyl-oxy)-peroxy ferulyl]-3-methoxy-salicylic acid

To assign the structure as a peroxy ester is absolutely based on the support of LC/MS and MS-2 data. This compound having high molecular mass and moderate polarity compared to compounds corresponding to peak no 2 and 3. Besides, having a central core of a ferulic acid with possible peroxy ester formation and therefore, might be responsible for the ethyl acetate fraction to show marked cytotoxic activity. Though there is no chemical evidence which could confirm the structure as 4[4-(3,4-dihydroxyphenyl-oxy)-peroxy ferulyl-3-methoxy-salicylic acid as a peroxy ferulyl ester but no alternative structure was possible because ^{1}H NMR clearly showed two extra doublets due to possibily of 3-methoxy–salicylic acid. If an extra oxygen function is placed in the 3-methoxy–salicylic acid, we get isolated singlets rather than two doublets which therefore strongly supports our speculation for the assigned structure besides as already discussed the strong evidence of mass fragmentation.

CHAPTER 4

PART II

IN VITRO CYTOTOXIC STUDIES

Material and Methods

Antibiotics

Penicillin Solution

6.25 mg of Penicillin (100 IU/ml) was dissolved in 100 ml of medium before use.

Gentamycin Solution

5 mg of gentamycin was dissolved in 100 ml of medium.

Other reagents

Phosphate Buffer Saline (PBS)

Dissolved contents of a vial of PBS in distilled water and diluted upto 1 litre.

Trypsin-EDTA

50 mg Trypsin (0.05%) and 20 mg EDTA, disodium salt (0.02%) were dissolved in PBS and diluted upto 100 ml.

TCA

50% (w/v) TCA solution was prepared in double distilled water.

Acetic acid

Glacial acetic acid was diluted to 1% with double distilled water.

SRB Dye

Dissolved 400 mg SRB (0.4%) in 1% acetic acid and diluted upto 100 ml.

Tris-buffer

1.21 g of Tris (10 mM) was dissolved in 950 ml distilled water; pH was adjusted to 10.5 and diluted upto 1 litre.

Growth media for cancer cell lines

(a) Incomplete growth medium

RPMI-1640 with 2 mM L-glutamine medium or MEM (contents of the vial) was dissolved in double distilled water (DDW) as per supplier's instructions. Streptomycin (100 mg ml^{-1}) was also added to medium. The pH of medium was adjusted to 7.2 and it was sterilized by filtering through 0.2 μ filters in laminar flow under sterile conditions. The media was stored in refrigerator (2-8°C).

(b) Complete growth medium

The incomplete growth medium was supplemented with 10% FCS and Penicillin (100 IU/ml, before use) to make it complete growth medium.

In vitro cytotoxicity against human cancer cell lines

Cell culture methods

Human cancer cell lines were grown in tissue culture flasks in complete growth medium (RPMI) at 37°C in an atmosphere of 5% CO_2 and 90% RH in a carbon dioxide incubator. The medium was changed by fresh medium from time to time as per requirement. The fresh medium was brought to 37°C in a water bath. The medium of the flask was taken out and discarded and fresh medium was placed. The volume of the medium used was dependent upon the capacity of the flask as per manufacturer's instructions.

Subculture of the cell lines

For subculturing, the medium of the flask having subconfluent growth was changed one day in advance. The entire medium from the flask was taken out and discarded. Cells were washed with PBS. Enough (0.5 ml) of Trypsin-EDTA in PBS (pre warmed at 37°C) was added to make a thin layer on the monolayer of the cells. The flask was incubated for approximately 5 minutes at 37°C and observed under microscope. If cells were found to be detached, complete growth medium (1.0 ml, pre warmed at 37°C) was added. Cell suspension was made. An

aliquot was taken out; cells were counted and checked for viability with trypan blue. Cell stock with more than 98% cell viability was accepted for determination of *in vitro* cytotoxicity. The cell density was adjusted to $1x10^6$ cells/ml by the addition of more complete growth medium and inoculated in two fresh TCF-75 and incubated in CO_2 incubator to continue the culture.

Preparation of test material

Stock solution

A stock solution of 20 mg ml^{-1} of the test material in DMSO was prepared.

Working solution

The stock solution were serially diluted to obtain working test solutions with complete growth medium containing 50 $\mu g ml^{-1}$ of gentamycin to obtain working test solutions of double the final concentration as per the requirements. The working test solutions were not filtered/ sterilized but microbial contamination was controlled by addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

Cytotoxicity assay

In vitro cytotoxicity against human cancer cell lines was determined (Monk *et al.*, 1991) using 96-well tissue culture plates. The cell suspension of required cell density was prepared in complete growth medium with gentamycin for determination of cytotoxicity. The aliquots of 100 µl of cell suspension were added to each well on a 96-well tissue culture plate. The blank wells contained complete medium in place of cell suspension. The cells were incubated for 24 hours.

Test material (100 µl in each well) of desired concentrations was added after 24-hours to the wells containing cell suspension and blank wells. Simultaneously, control experiments with and without suitably diluted DMSO in place of test material, and positive controls containing known anticancer agents were also carried out. All the experiments were carried out in triplicate.

The cells were allowed to grow in presence of test material by further incubating the plates for 48 hours. At the end of incubation period the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50µl/well) on top of the medium in all the wells. The plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells. Supernatant of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc and air-dried.

Sulphorhodamine B (SRB) assay

The SRB assay was perfomed to assess cell growth (Skehan *et al.*, 1990). SRB (100 µl/well) was added to each well and plates were allowed to stand at room temperature for 30 min. Then the plates were washed four times with 1% acetic acid. The plates were dried and Tris-buffer (100 µl/well) was added to each well to solublise the dye. The plates were shaken gently for 10 minutes on a shaker and the optical density was recorded on ELISA reader of Robotic liquid handling system at 540 nm.

Calculation

The cell growth was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.

Effect of fungal CPT on morphology of human cancer cell lines

HL-60cells were centrifuged at 2000 rpm for 10 min and were spread over glass slides and allowed to air dry. The air dried smears were fixed in absolute methanol for 2 min and stained with Giemsa. The Hep-2 cells grown on cover slips were subjected to light microscopic studies.

In order to ascertain the mechanism of action of fungal CPT on human cancer cell lines, the light microscopic studies of HL-60 and Hep-2 cells after treatment were done. The photography was done using Olympus digital camera attached to light microscope (VANOX).

Screening of organic extracts of endophytic fungus for *in vitro* cytotoxicity against human cancer cell lines

The chloroform: methanol extract of mycelia (6 g) was prepared as described earlier (chapter-3) in the present study and was subjected to column chromatography (using 500 g silica gel, 60-120 mesh size) and the graded elution was carried out with benzene, ethyl acetate, chloroform and methanol. After proper elution, the fractions which showed identical pattern on TLC were pooled and thus designated as fractions, CHF-1 (benzene fraction), CHF-2 (ethyl acetate fraction), CHF-3 (chloroform fraction) and CHF-4 (methanol fraction).

Butanolic extraction of marc

The marc obtained after extraction of fungal mycelia with chloroform: methanol (4:1) was further extracted with n-butanol (70%). The butanolic layer was concentrated, dried and designated as fractions, CHF-5.

All the extracts/fractions were studied for their *in vitro* cytotoxicity against human cancer cell lines as described earlier on page 126-128. The results are summarized in Table 11.

Results

In vitro cytotoxicity of fungal CPT and plant CPT against human cancer cell lines

In vitro cytotoxicity of CPT isolated from the plant and fungus was studied against six human cancer cell lines from four tissues at four concentrations from $1x10^{-8}$ to $1x10^{-5}$ (Table 10). A concentration dependant growth inhibition was observed against all the cell lines. The degree of growth inhibition was cell specific. The HCT-15 cell line of the colon showed maximum inhibition where as SW-620 cell line was least sensitive. However, the CPT of both the origins were found to be equally effective.

Effect of fungal CPT on morphology of human cancer cell lines

In order to study the mechanism of action of CPT on human cancer cell lines, the light microscopic studies of untreated and CPT treated HL-60 and Hep-2 cells were done. The untreated HL-60 cells afre spherical in shape and have large sized nuclei leaving cytoplasm only to the periphery (Fig. 42 a-b). After treatment, the cell showed apoptotic morphology such as reduction in size, condensation of nuclei and vacuolisation of cytoplasm (Fig. 42 c-d). The nuclear fragmentation was also seen (Fig. 42 c-f).

Untreated Hep-2 cells are polymorphic in shape and showed numerous protoplasmic extensions (Fig. 43 a, c). The treated cells showed reduction in size, condensation of nucleus and the protoplasmic extensions were reduced. All these characteristics are found in apoptotic cells (Fig. 43 b, d).

Screening of organic extracts of endophytic fungus for *in vitro* cytotoxicity against human cancer cell lines

In vitro cytotoxicity *of* fractions/extracts was determined against 10 human cancer cell lines, from liver, colon, CNS, prostate, oral, lung tissues at two concentrations i.e. 10 and 30 μgml^{-}. 5-Flurouracil ($2x10^{-5}$M), Mitomycin and Paclitaxel ($1x10^{-5}$ M), were used as references (Table 11). The methanol fraction, benzene fraction and butanol extract of marc did not show any appreciable activity at both

concentrations and were inactive. However, the ethyl acetate fraction showed significant growth inhibition against all the cell lines. The maximum inhibition was observed against Hep-2 (Liver) cell line. The other cell lines such as SW-620, HCT-15, HT-29, DU-145. LN amd A-549 also showed more than 50% growth inhibition at 10 μgml^{-1}.

Table 10: *In vitro* cytotoxicity of fungal CPT and plant CPT against human cancer cell lines

Growth inhibition (%)												
Tissue	Colon				Liver		Lung				Ovary	
Cell lines	HCT-15		SW-620		Hep-2		A-549		HOP-62		SK OV-3	
Conc. (M)	Fungal CPT	Plant CPT	fungal CPT	Plant CPT	Fungal CPT	Plant CPT	fungal CPT	plant CPT	Fungal CPT	plant CPT	fungal CPT	Plant CPT
$1x10^{-8}$	32	37	1	0	14	13	24	23	19	19	10	17
$1x10^{-7}$	42	42	21	23	62	54	37	34	30	28	15	18
$1x10^{-6}$	59	71	39	43	69	67	47	37	33	27	25	32
$1x10^{-5}$	84	81	42	58	71	78	67	52	41	33	48	50

The *In vitro* cytotoxicity was determined as per method mentioned at page 126-128. The values reported here in are the mean value of two experiments each carried in triplicate.

Table 11: *In vitro* cytotoxicity of organic extracts/fractions of fungal mycelia against human cancer cell lines

Growth inhibition (%)											
Fractions from Chloroform: methanol (4:1) extract of mycelia	Tissue	CNS	Colon				Liver	Lung		Oral	Prostate
	Cell lines	SNB-78	SW-620	HCT-15	502713	HT-29	Hep-2	A-549	H226	KB	DU-145
	Conc. (μgml^{-1})										
MeOH fraction (CHF-4)	10	9	3	14	0	0	17	26	27	6	7
	30	14	0	10	2	0	19	27	43	2	12
Benz fraction (CHF-1)	10	10	0	12	0	0	18	27	39	9	11
	30	0	0	13	0	0	14	28	40	0	17
Ethyl acetate fraction (CHF-2)	10	22	53	70	33	63	93	66	45	85	62
	30	25	56	83	46	86	93	67	48	86	56
Butanol extract of marc (CHF-1)	10	12	0	0	2	0	13	30	37	12	15
	30	10	2	8	17	0	20	30	43	0	19
5FU	$2X10^{-5}$ M		17	28	19	30					
Mitomycin C	$1X10^{-5}$ M						68			90	
Paclitaxel	$1X10^{-5}$ M	46						63	60		85

The *In vitro* cytotoxicity was determined as per method mentioned at page 126-128.
The values reported here in are the mean value of two experiments each carried out in triplicate.

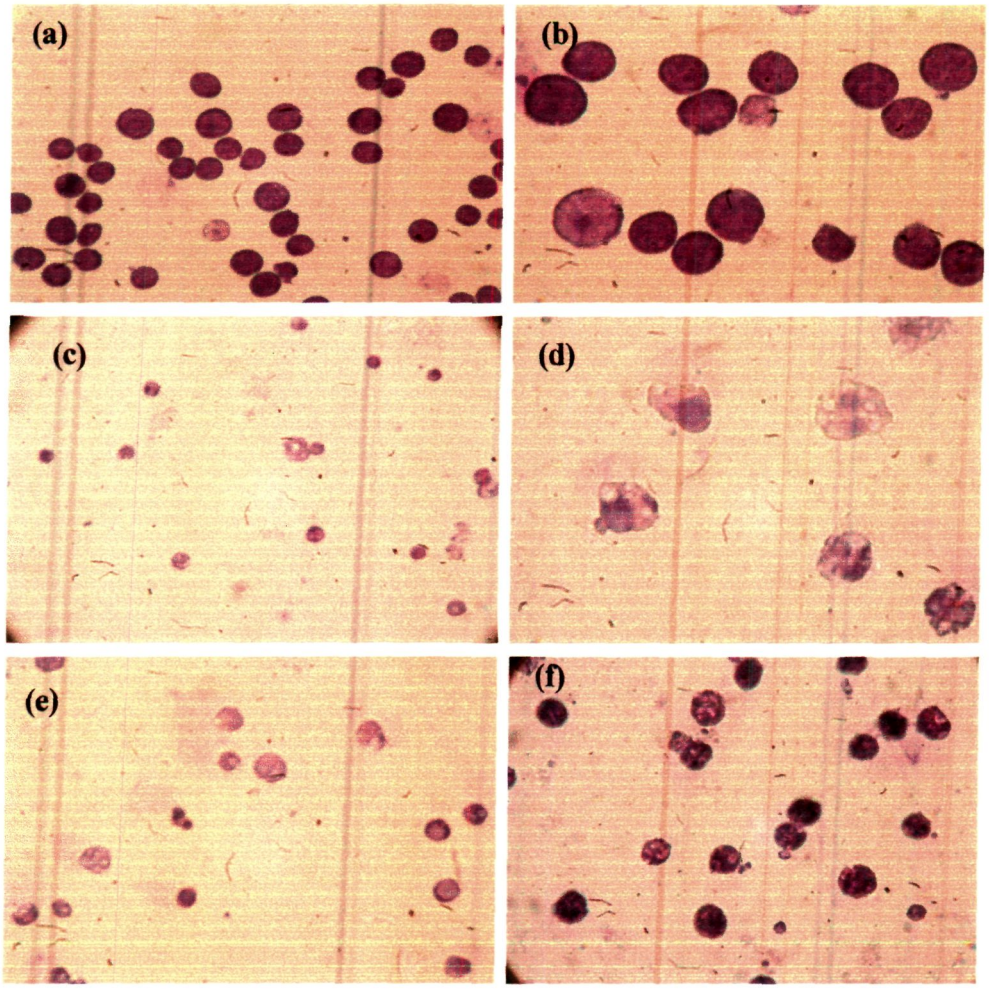


Fig. 42:(a-b) Untreated HL-60 Cells, (c-d) Doxorubicin treated HL-60 cells, (1x10-5 M) show condensation of cytoplasm and nucleus, (e-f) CPT treated HL-60 cells also show condensation of cytoplasm and nucleus. (a, c and e Mag. 450X, b, d and f Mag.900X)

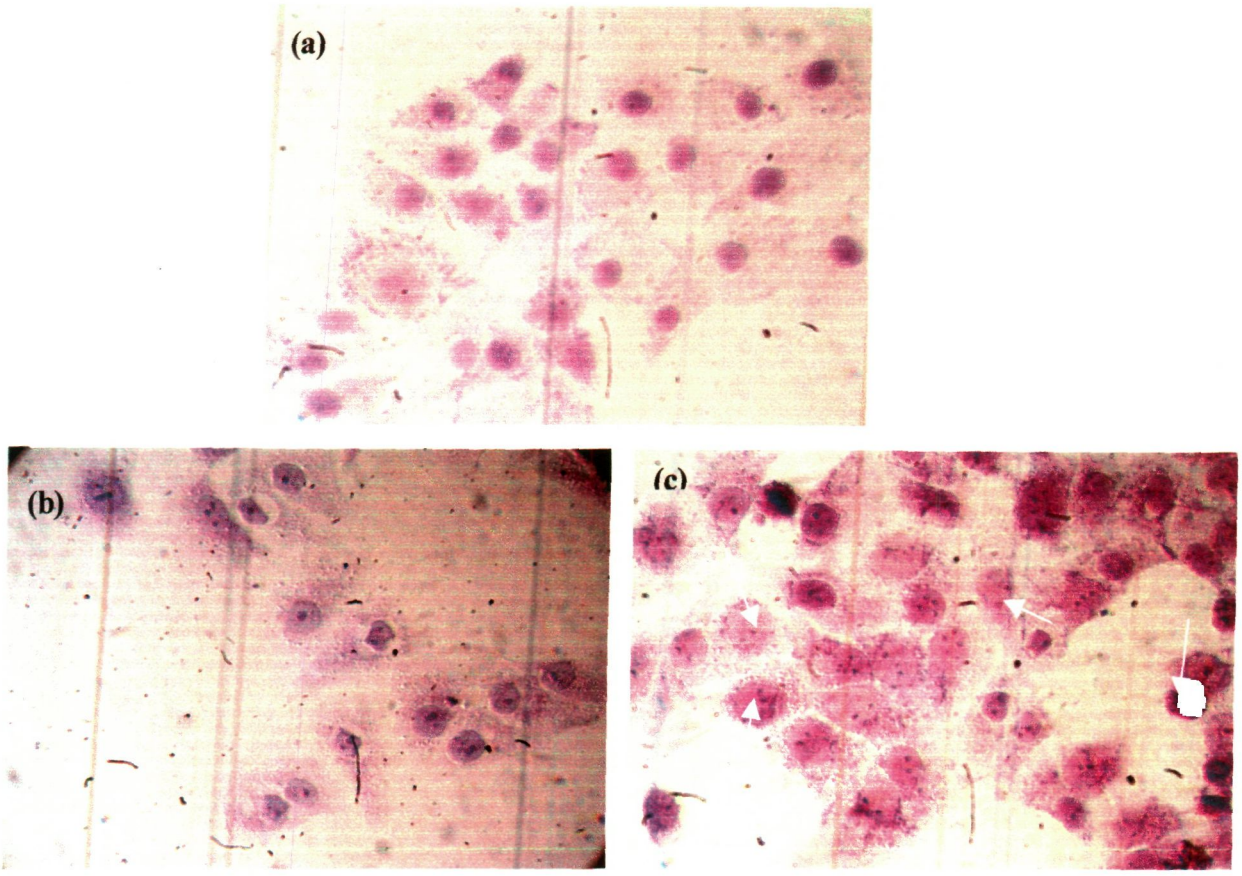


Fig. 43: (a-c) Hep-2 cells. (a) Untreated, (b) Mitomycin-C treated cells. Mag. 450X (c) CPT treated Hep-2 cells ($1x10^{-5}$ M) show retraction of protoplasmic extensions, condensation and marginalization of chromatin material in the nuclei (arrow) Mag. 450X

CHAPTER 5

DISCUSSION

The genus *Entrophospora* was proposed by Ames and Schneider (1979) based on spores wet-sieved from a celery field in California, USA. They resembled spores of the incompletely described *Glomus infrequens* Hall (Hall, 1977), although the authors noted similarities with *Acaulospora*, the spores formed inside the vesicular stalk (sporiferous saccule) rather than laterally (Ames and Schneider, 1979). The species was given the name *Entrophospora infrequens* (Hall) Ames and Schneider and established as the type species for the new genus, *Entrophospora* Ames & Schneider, which superseded *Glomus infrequens*. The genus *Entrophospora* currently includes four other species, *E. colombiana* Spain & Schenck (Schenck *et al.*, 1984), *E. schenckii* (Sieverding and Toro, 1987), *E. kentinensis* (Wu & Liu, 1995) and *E. baltica* (Blaszkowski *et al.*, 1998). The low number of *Entrophospora* species may be the result of a small or regulatory change that converted each *Acaulospora* to an *Entrophospora* under rare circumstances (either recently or over several hundred million years). The few DNA sequences of rDNA published thus far (Simon *et al.*, 1993) suggest a monophyletic origin, but these sequences are so highly conserved that the low number of taxa and absence of relevant comparisons could be misleading. The selected organisms identified, as *Entrophospora infrequens* by LSU rDNA typing in this study was isolated from inner bark tissue of *Nothapodytes foetida* plant at Regional Research Laboratory, Jammu, hitherto reported as ectomycorrhizal fungi.

Twigs (young and old) from *Nothapodytes foetida* plant (Fig.13 b) growing in Jammu and Mahabaleshwar regions in India were subjected to the isolation of fifty two colonies of endophytic fungi (Fig.15) and were tested for their ability to produce the anticancer alkaloid camptothecin. One of the isolates (RJMEF001) from the inner bark tissue of the *N. foetida* plant growing in Jammu region of J&K state, India was found to produce detectable quantities of camptothecin and its derivatives when grown in a semi-synthetic liquid medium. The search for *N. foetida* associated microbes that produce Camptothecin is justified by previous examples of plant associated microbes producing plant compounds, such as gibberellin (Stowe and Yamaki, 1957). The pathways of gibberellins biosynthesis in the fungus and the higher plant are identical up to GA_{12} (Lang, 1970). Each one

of the five classes of plant hormones (auxins, abscisins, ethylene, gibberellins and kinetins) is, in fact, known from a wide range of representative plant-associated fungi and bacteria (Goodman *et al.,* 1986). Herein, we report for the first time, the production of quinoline alkaloid camptothecin by an endophytic fungus (RJMEF001) isolated from *N.foetida* (Puri *et al.,* 2005c), a plant from Kokan Ghats in India and presently being maintained in the botanical gardens of Regional Research Laboratory, Jammu.

Media manipulation to alter the fungal metabolites

Microorganisms often produce secondary metabolites in response to environmental stress or nutrient availability. Stress can be chemically induced by physico-chemical manipulations, which can result in the production of unique metabolites not ordinarily associated with a particular microorganism. Both unusually high and low levels of particular nutrients can alter fungal metabolism. These chemical stresses can result in highly reproducible yields of unusual natural products. Endophytic fungi offer a unique opportunity for media alteration because endophytes may metabolize certain classes of nutrients produced by their hosts. Removal from the host can alter secondary metabolite production, so the addition of host by products may be equally effective. This metabolic response to specific chemical cues is bioenergetically sound. Varying the fermentation protocols in the synthetic media can also greatly affect the production and diversity of secondary metabolites. Adding high levels of one nutrient or trapping another nutrient may result in substantial differences in secondary metabolite production. (Tanaka, 1992). Productivity amplification is relatively easy in microorganisms. In the case of penicillin, improved culture conditions and genetic manipulation of developing strains of penicillium increased drug yield from a few micrograms per milliliter to thousands of micrograms per milliliter (Demain, 1961; Grayson, 1982). Different bioactive compounds can be produced by altering culture conditions. The antibiotic aplasmomycins was produced by *Streptomyces griseus* SS-20 only after the addition of NaCl to the medium (Okami *et al.*, 1976). Directed changes in the culture conditions can be explored

indefinitely as a means of optimizing biosynthetic pathways that may lead to even more effective analogues (Okami, 1986). With this background, in the present study, the production of secondary metabolite (camptothecin) of *N. foetida* associated endophyte- RJMEF001 in response to the media manipulation was examined. The production of CPT from endophyte- RJMEF001 was carried by submerged fermentation in shake flasks. *E. infrequens*-RJMEF001 can be propagated on several economically viable simple and synthetic media, viz.; Czapek, Malt extract, Molasses, Goos and Tschessch, Potato Dextrose, Ashner, and Kohn, Leonine, Bianchi, and Sabouraud broth. Sabouraud broth was found to be the best medium, among the various growth media tried for the production of camptothecin. The optimum temperature and pH being 28±2° C and 5.6 respectively. The medium containing 0.62% (w/v) maltose and 0.06% (w/v) peptone, 0.62%(w/v) malt extract as sole carbon and nitrogen source resulted in lowest camptothecin production (200.34 ± 19.95µg/100 g dry cell mass). However highest CPT production of 503.07 ± 25.88 µg/100 g dry cell mass was obtained in sabouraud broth in combination with 1% (w/v) peptone and 4% (w/v) Dextrose (DMH). On the basis of CPT production and biomass estimation, it was concluded that good growth but no CPT production was obtained on Czapek (M_1, M_2, M_{13}, M_{14}) and malt extract (M_5) media. Neither CPT nor good biomass production was observed in high osmotic stress medium (M_{12}, M_{15}, M_{16}). A range of carbon and nitrogen sources was screened for their capacity to support growth of *E. infrequens* and CPT production. Suppression of CPT production by the presence of disaccharides and polysaccharides like sucrose and starch as sole carbon sources was observed. However, comparatively good biomass and increased CPT production was obtained with glucose monohydrate. Peptone was found to be effective nitrogen source for the production of camptothecin.

Growth and production kinetics at shake flask level and in fermenter

The production of camptothecin from *E. infrequens*- RJMEF001 was carried out by submerged fermentation in shake flasks as well as at bench fermenter scale.

Sabouraud medium was found to be the best medium, among various growth media tried for CPT production. The growth kinetics of the endophyte (under the standardized culture conditions described in Table.6), which exhibited an exponential increase in dry weight of the mycelia (Fig.19 a), up to seventh day of incubation was examined. In order to have an insight in the production kinetics of CPT, the mycelia were collected every 24 hours and CPT was isolated. The cell homogenates thus obtained, were extracted using different solvent systems. Out of the different solvent systems tested, chloroform: methanol (4:1 v/v) exhibited better seperation of an organic residue in rotary evaporator with a compound having besides many other compounds, the same chromatographic mobility as for authentic CPT. As the camptothecin production was observed to start 24 h after incubation and reached maximum of 0.575 ±0.031 mg/100 g dry cell mass and 4.96 ± 0.73 mg /100 g dry cell mass at 96 h and 48 h of cultivation in shake flask and bioreactor level respectively (Fig.19 b & 20 d). The fermenter was operated under continuous shaking (200 rpm) and acidic pH (5.6) of the culture broth. The dissolved oxygen and final pH at the time of termination were recorded as 56% and 2.5, respectively. The optimum temperature and rpm for maximum CPT production were found to be 28 ± 2°C and 200, respectively. High CPT production was observed when *Entrophospora infrequens* was grown in Sabouraud broth with 1% (w/v) peptone and 4% (w/v) glucose monohydrate. It has recently been observed that the monoterpene-secologanin moiety of the CPT is synthesized via 2C-methyl –D-erythritol-4-phosphate (which is generated from glucose) pathway not via the mevalonate pathway (Yamazaki *et al.,* 2004). Preliminary results (Amna *et al.,* 2006) have been very encouraging. Further refined studies in large-scale fermenter are likely to result in an efficient process for production of secondary metabolites (CPT) of *E. infrequens* in large quantities that could be utilized for industrial production of this important group of compounds. The results indicate that *Entrophospora infrequens* is an excellent candidate for consideration in fermentation technology. *Entrophospora infrequens* may be potential organism for further development and optimization of a

fermentation process for the production of camptothecin and its derivatives as an alternate source to *Nothapodytes foetida* roots.

Stimulation of the production of CPT by biogenetic precursors and elicitors in fungal culture

Alkaloids form one of the most widely studied groups of plant secondary metabolites; over 12000 alkaloids have been characterized from the plant kingdom (Facchini, and Bird, 2004). Tryptophan (Leete, 1961; Kutney *et al.*, 1968, 1971), tryptamine (Kutney *et al.*, 1968; Battersby *et al.*, 1968a, 1969a), mevalonate (Leete *et al.*, 1962; McCapra *et al.*, 1965; Battersby *et al.*, 1966a; Hall *et al.*, 1966; Money et al., 1968), geraniol (Hall *et al.*, 1966; Money et al., 1966; Battersby *et al.*, 1966b; Loew *et al.*, 1966; Leete and Ueda, 1965; Escher *et al.*, 1970), loganin (Battersby *et al.*, 1966c; Battersby *et al.*, 1970), secologanin (Battersby *et al.*, 1969b) and vincoside (Battersby *et al.*, 1968a, 1969a), have already been established as precursors of CPT type of indole alkaloids. Sheriha and Rapoport (1976) have shown with their experiments that Tryptophan, mevalonic acid, nerol, and geraniol are incorporated in the biosynthesis of camptothecin in the plant *Camptotheca acuminata.* With the background about the biosynthesis of CPT in plant, fungal culture was fed with the same precursors. Our observations are in variance with their observations. Though tryptophan without any doubt is the main precursor for the synthesis of the alkaloid camptothecin so far as ring A, B and partly ring C is concerned (Fig. 1a), however our experiments showed that the incorporation of terpenoid portion of the alkaloid involving ring D and E is concerned geraniol, nerol and even surprising mevalonic acid do not appear to be the precursors of the skeleton. Mevalonic acid is an established precursor for the biosynthesis of mono, sesqui and triterpenoids but we failed to agree with the above-mentioned authors where they have shown the incorporation of these in the biosynthesis. Our results have shown that tryptophan or tryptoamine do enhance the yield of the alkaloid as expected but for the monoterpenoid part of alkaloid our results (Table 7) have shown that leucine (0.025M) along with Tryptophan (0.01M) does increase the yield of CPT. This

clearly indicates that Leucine is the precursor for the terpenoid portion of the alkaloid i.e. ring D and E portion of the alkaloid. Data represents mean of three replicates and the experiment was repeated three times to get reproducible results. These experiments open new vistas for the study of biosynthesis of such alkaloids where labelled precursors are to be used for the biosynthesis of fungal metabolites.

Production of CPT by endophytic fungus by Solid-state fermentation and under surface culture conditions

CPT was produced by endophytic fungus *E.infrequens* by surface culture and solid-state fermentation and their physiological and kinetic properties were studied. The type of culture method used influenced the kinetic and physiological properties. The highest CPT production of 3.37±0.44 mg/100 g dry cell mass with cell mass of 22.79 ± 0.8 gl^{-1} was obtained with surface culture fermentation in conical flasks at 21 days of incubation time in sabouraud broth. The composition of the production medium is key parameter in optimizing SSF process also because nutritional factors such as the carbon source and the levels of nitrogen and trace metals, can influence the growth and production of metabolites, (Guttierrez-Rojas *et al.,* 1995) enzymes, (Christen *et al.,* 1995) and spores (Larroche, 1996). The use of supports impregnated with liquid media offers additional flexibility when designing the medium for the optimal production of metabolites, enzymes and spores. In the present study, the results obtained during solid-state fermentation on moist wheat bran and wheat bran supplemented with sabouraud broth indicated maximum productivity of 200.33 ±19.90 µg/100 g dry weight and 390.78 ± 20.10 µg/100 g dry weight respectively at 21 days. The less prominent difference in the production was obtained. However, the production under surface culture conditions is approximately nine folds higher than the production by solid-state culture. One of the differences between SSF and surface cultures is that in the former the moisture content of the substrate is low, resulting in a limitation of the growth and metabolism of microorganism. The concept of water availability in a substrate thus becomes very important. The results in this

work suggests that culture method (surface culture or solid state) may be responsible for inducing changes in CPT content produced by *E. infrequens*.

Isolation and quantification of camptothecin in fungus by LC-MS/MS

This method describes the detection of Camptothecin (CPT) in *N. foetida* associated endophytic fungus and application of gradient reverse phase HPLC method with diode array and MS^2 detection for the quantification of said compound. The quantity of CPT in the extracts were estimated on the basis of linear calibration curves obtained in the concentration range of 5 to 50ng with standard CPT. Fungus grown under surface culture method accumulated 40mg CPT/kg dry cell mass which is far lower than that present in the plant source (Govindachari and Vishwanathan, 1972a) from where the organism was isolated. However, this is the first such report on the accumulation of CPT in a fungus and may be a starting point for improving the productivity of CPT in this isolate. In order to support such studies there is a need for development of a very sensitive and effective assay technique to quantify the production of CPT in mycelium extracts and fermentation broth. Here, in the present study we report the quantification of camptothecin by LC-MS/MS. This method has the advantage over LC-UV (DAD) and LC-MS methods because of its selectivity and specificity. Binary gradient reverse phase HPLC with diode array absorbance and MS^2 detection in the +ve mode of ionization under Electro spray API interface in a Single Reaction Monitoring (SRM) system was used for detection and quantification of camptothecin.

Chemical constituents of *N.foetida* associated endophytic fungus RJMEF001

Investigation of the bioactive components of the endophytic fungi associated with the *N.foetida* plants yielded a novel fungus that produced CPT and its derivatives when grown in semi synthetic liquid media. *Entrophospora infrequens* was isolated from inner bark of the *N.foetida* twigs. The presence of CPT in the

fungal extract after detailed chromatographic separation and purification was confirmed by various physico-chemical properties and cytotoxic studies. Both tryptophan and leucine appeared to be good precursors of CPT in fungal culture. But *E. infrequens*-RJMEF001 is neither a robust organism nor a high level CPT producer. This first discovery merely spurred the search for other, more viable CPT producing endophytes. The ultimate purpose of this study was to isolate novel, active metabolites with drug potential. Although a few such compounds have surfaced from this work. The organism was found to be rich source of ergosterol. The ergosterol has previously been isolated from a variety of fungi, including Basidiomycetes, *Fungi imperfecti* (Goad, 1967), yeast (Goad, 1967) Zygomycete, *Blakeslea trispora* (Goad, 1966) and Mucorales (McCorkindale *et al.*, 1969).

The ergosterol content reported in the literature for *Aspergillus, Penicillium, Fusarium, Rhizopus, Cladosporium, Candida* and *Alternaria* species ranged from 0.4 to 14.3 µg /mg (Axelssen *et al.,* 1995; Schnurer, 1993; Seitz *et al.,* 1979). Interestingly; the ergosterol content was found to be fairly high and is in the range of 16-18µg/mg. Moreover, the bioguided fractionation of ethyl acetate fraction of mycelia yielded three molecules viz., (1) 4-(3, 4-dimethoxy-phenyl-1-oxy)-caffeic acid (2) 4-(3,4-dihydroxy phenyl-1-oxy)-ferulic acid and (3) 4[4-(3,4-dihydroxyphenyl-oxy)-peroxy ferulyl]-3-methoxy-salicylic acid which were found new to the literature. Besides, the organism also produced 5-(hydroxymethyl)-2furfuraldehyde in culture. Recently Kim *et al.,* (2005) have shown the presence of 5-Hydroxymethyl-2-furfuraldehyde present in *Euphoria longana* L. as an important anticonvulsant furan. It is interesting therefore, to find 5-Hydroxymethyl-2-furfuraldehyde in the endophytic fungus isolated from *N. factida* plant. This may help to emphasize the use of fungus as an important alternate source of such compounds.

In summary, we report for the first time to the best of our knowledge, the production of camptothecin by an endophytic fungus. Camptothecin and its analogues are naturally occurring group of quinoline alkaloids depicting profound cytotoxic activity (Bodley *et al.*, 1998). Various plant species such as *Camptotheca acuminata, Ophiorrhiza mungo, Ervatomia hyneana and*

Nothapodytes foetida are known sources of this photochemical (Aiyama *et al.*, 1988). The supply of CPT depends primarily on the abundant availability of plants such as *Camptotheca acuminata*. Many parts of this plant can be used to extract CPTs (Li and Adair, 1994). The overexploitation of this source rendered this plant as an endangered species all over the globe especially in China. The gene pool of this plant is very small in countries like USA (Li *et al.*, 2000b). Literature survey on camptothecin revealed that molecule occupies an important position among the plant based anti cancer drugs. In order to conserve the germplasm, a need is felt to look for alternate sources for this class of natural products. The discovery that fungi can bio-synthesize camptothecin, increasingly lends support to the possibility of horizontal gene transfer between *N.foetida* and its corresponding endophytic organism. The consistent production of camptothecin and its analogues by *E. infrequens* further supports the theory of Young and co-workers (Young *et al.*, 1992) that during the course of evolution, the symbiotic endophytes developed machinery to biosynthesize and tolerate high levels of secondary metabolites in order to better compete and survive in association with the medicinal plant.

In this study, the biological importance of the fungal originated camptothecin possessing anti-cancer activity was also established. Biological activity of fungal camptothecin was assayed *in vitro* (Skehan, *et al.*, 1990) against human cancer cell lines (A-549) for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer and compared with standard CPT (10^{-5}M). A comparable cytotoxic activity profile was observed. The methanol, benzene and ethyl acetate fractions from mycelia collected on Day 4 were also subjected to *in vitro* cytotoxicity (Table 11). The ethyl acetate fraction was found to be most active and rest of the fractions showed only very low or no activity. The butanol extract of mycelia collected on day 4 was also analyzed for its *in vitro* cytotoxicity and was found to be devoid of any activity. Previous workers have reported the production of the anti-leukemic and antitumor drug taxol from the endophytes of *Taxus* spp. like *Taxomyces andreanae* and *Pestalotiopsis microspora* (Stierle *et al.*, 1993; Strobel *et al.*, 1996). Others workers have reported the production of anti-fungal compounds by endophytes (Liu *et al.*, 2001).

Earlier workers have reported the role of mycorrhizal fungi in uptake of nutrients, tolerance of water stress and adverse environmental conditions, erosion control. Others have also suggested that mycorrhizae (Dodd, 2000) play a crucial role in protecting roots from heavy metals like Cd, Pb, Zn etc. (Galli *et al.*, 1994; Marschner, 1995). In addition, micropropagated plantlets inoculated with mycorrhizal fungi particularly *Entrophospora* spp. have better survival rate than un-inoculated plantlets and also increased *podophyllum* yields with increments of growth and lignan content (Moraes *et al.,* 2004). The present study has focused only on the production of camptothecin by *E. infrequens*, however, the implications of the present study in environmental biotechnology particularly stresses associated with nutrition, water/aeration, soil structure, pH, salt, toxic metal, and pathogens remains a virgin area that needs to be explored. The next step will be to expand and apply the work at various levels to other areas of biotechnology. We expect that biotechnological application of the endophyte isolated will be in multiple areas.

SUMMARY

The thesis embodies the work on the chemical investigation of fungal endophytes harboured in medicinal plant, *Nothapodytes foetida* (Icacinaceae), native to Western Ghats. It includes a short review of literature of various constituents isolated from different endophytes. It also includes the isolation of camptothecin, a naturally occurring alkaloid and an important precursor of anticancer drugs, from plant as well as from a new source "endophytic fungus." Furthermore, it also describes the isolation of two known and three new metabolites from this novel endophyte. Manuscript of the thesis comprises of five chapters.

The first chapter of this dissertation presents general introduction about fungal endophytes and an overview of different facets investigated in this study with emphasis on the natural precursor of anticancer drug camptothecin. The second chapter has been devoted to the summary statement of a review on few representative groups of fungal endophytes, their associated secondary metabolites and a chemical and biological profile of some important leads. In addition, a brief description of diversity of fungal endophytes has also been given.

The third chapter, which forms the major part of the thesis, deals with the isolation of endophytes from *Nothapodytes foetida* plants obtained from various locations and biological studies of a selected fungal endophyte-RJMEF001, later on identified as *Entrophospora infrequens*. Even when the present investigations have resulted in the isolation of a number of endophytic fungi from the *Nothapodytes foetida* plant, the selected fungus is reported here to produce camptothecin and its analogues when grown in semi-synthetic liquid medium. Besides, detailed shake flask studies under different culture conditions have been discussed. In addition to that optimization of media and identification of the organism, using different microscopic as well as molecular techniques have also been discussed in detail. Further, preliminary precursor and solid-state fermentation studies have also been described. This chapter includes the scale up studies for the biomass as well as camptothecin production in bioreactors.

The fourth chapter deals with the experimental details and the spectral data of the constituents isolated from the different solvent extracts of mycelia of *E. infrequens*. Further more, an attempt has been made to assess the *in vitro* cytotoxicity of fungal extracts made by organic solvent extraction, chloroform:

methanol and butanol as well as the fractions eluted in benzene, ethyl acetate, and methanol. The *in vitro* cytotoxicity of the fungal extracts evaluated using 10 human cancer cell lines (Hep-2: liver, SW-620, HCT-15, 502713, HT-29: colon, SNB-78: CNS, DU-145: prostate, KB: oral, A-549, H226: lung) at 10 and 30 μgml^{-1} doses is also described as a bioassay in support of the qualitative presence of CPT. An elaborate use of chromatography and spectroscopy has been made for the isolation and structure determination of the compounds. The present investigations have resulted in isolation of six molecules such as camptothecin (CPT), 5-(hydroxymethyl)-2-furfuraldehyde, and ergosterol along with three other compounds, the structure of which indicates the novelty of these compounds to this fungus. The discussion on the entire set of results and the thesis emerging out of such results constitute the last chapter. At the end of the thesis, references to the relevant literature have been incorporated. Following is the summary of the work accomplished.

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial arena. Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism, product and utilitarian discovery in this field holds exciting promise. This is witnessed by the discovery of a wide range of microorganisms and the products thereof indicating these organisms as a promising resource for prospecting new chemical entities as future drugs.

The use of endophytic fungi for the production of secondary metabolites has been a priority regarded as an area of considerable interest, owing their enormous potential for production of bioactive compounds. The present study is based on the premise that screening of beneficial endophytic fungi possessing enhanced anticancer capabilities in conjunction with some additional traits viz. immunomodulators, antiviral, antibacterial, antifungal and radio protective compounds production may help provide certain strains with broad spectrum activities. The extended significance of the productivity of endophytes for these important bioactive metabolites lies in that, they provide an alternate strategy for

easing the negative impact of over exploitation of resource plant which are otherwise essential part of biodiversity and the ecosystem. As a poorly investigated store of microorganisms, hidden within the host plants, these endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with substantial medicinal and agricultural potential. Thus, the isolation, characterization and identification of such indigenously effective fungal flora with broad-spectrum biological activities are perceived as a viable and cost effective approach for developing a genetic resource.

In this study, the indigenous fungi have been isolated from wild *N. foetida* plant samples collected from Mahablashwer forest and grown successfully at RRL botanical garden of Jammu province (32^0 44 N' and 74^0 55' E, approximately 400m, altitude above see level) of J&K state, India in the year 1995-96.This important medicinal plant was for the first time explored for harbouring endophytes. Using skilled isolation procedures, 52 different endophytic fungal colonies were isolated from inner bark tissues of *N. foetida* explants. These fungal colonies were purified by routine single spore isolation, sub culturing and hyphal tip methods. All the isolates were screened for the production of anticancer alkaloid camptothecin through chemical profiling as well as by exhibiting innate potential of biological activities. One of the isolates (RJMEF001) from the inner bark tissues of *N. foetida* plant growing at RRL, Jammu was found to produce detectable quantities of CPT and its derivatives when grown in semi synthetic liquid medium. The fungal strain was characterized on the basis of its morphological and 16S ribosomal genetic markers. The scanning electron microscopy was used to record the characteristic features of the fungal spores. It demonstrated the prominent striations on the smooth surfaces of fungal spores, which are round to oval in shape. The morphological and phylogenetic analysis based on LSU rDNA typing, comparing the sequences in NCBI and ribosomal data banks suggested the identification of the strain RJMEF001 as *E. infrequens.* Sequence alignment with database showed that the strain is more close to *E. infrequens* (>98%) than the other close taxa e.g., *Rhizopus oryzae* strains UW FP973 and 846 (>97%). On the basis of morphological features, growth behaviour as well as DNA alignment homology, the organism is assumed to be *E.*

infrequens. This promising isolate, *E. infrequens*-RJMEF001, was cultivated in various mycological broths viz; Czapek, Malt extract, Molasses, Goos and Tschessch, Potato Dextrose, Ashner, and Kohn, Leonine, Bianchi, and Sabouraud broth supplemented with different salts and trace elements and designated as M_1, M_3, M_6, M_7, M_9, M_{10}, M_{11}, M_{12} and M_{18} respectively throughout in the present study, in order to optimize the medium for maximum growth and production of camptothecin. Significant quantitative and morphological changes were detected when the fungus was challenged to grow in different liquid media. The strain RJMEF001 exhibited substantial growth i.e. 28.48 ± 1.08 gl^{-1} in Czapek medium supplemented with Dextrose 30 gl^{-1} as a sole carbon and energy source and ammonium oxalate 3.0 gl^{-1} as nitrogen-source. But there was no detection of camptothecin in this broth. The camptothecin (489.42 ± 19.87 µg/100 g dry cell mass) was detected in culture filtrates of the fungus when grown in Sabouraud broth supplemented with trace elements magnesium sulphate (0.5gl^{-1}) and potassium dihydrogen orthophosphate (1.0 gl^{-1}). The CPT was also detected in varied amounts in cultures grown in liquid media viz. Goos and Tschessch (326.42 ± 36.55 µg), Leonine (200.34 ± 19.95 µg), and molasses (230.16 ± 25.88 µg) per 100 g dry cell mass. A range of carbon and nitrogen sources was screened for their capacity to support growth of *Entrophospora infrequens* and CPT production. There was no CPT formation in medium containing malt extract (M_3, M_{15}, M_{16}), Czapek (M_1, M_2, M_{13}, M_{14}), Ashner and Kohn and Bianchi liquid broths also did not support CPT production. The lowest cell mass (1.84 gl^{-1}) was obtained in high osmotic stress broth (M_{15}) using 10% NaCl. However, only traces of CPT were detected in potato dextrose broth. The highest CPT production of 503.07 ± 25.88 µg/100 g dry cell mass was obtained in Sabouraud broth with 1% (w/v) peptone and 4 %(w/v) dextrose. Sabouraud broth was found to be the best medium, among the various growth media tried for the production of camptothecin. The results on the growth kinetics in Sabouraud liquid medium indicated maximum dry biomass of 28.11 ± 1.92 gl^{-1} after 7 days when incubated at 28 ± 2°C. The results on the production kinetics indicated the maximum production of 0.575 ± 0.73 mg/100 g dry cell mass in 96 h incubation period. The

generation time for the strain RJMEF001 was determined to be 14-16 h in sabouraud medium. Besides media optimization, the culture was also fed with known precursors of camptothecin. Analysis of the precursors data revealed the varied rates of CPT production in Sabouraud broth supplemented with different precursor molecules. Interestingly, the endophytic fungal strain showed enhancement in the CPT yield in Sabouraud broth supplemented with tryptophan (10 mM) and tryptophan in combination with leucine (25 mM). The CPT yields were almost increased by 2 fold (estimated as 871.58 ± 86.2 µg/100 g dry cell mass and of 993.68 ± 99.3 µg/100 g dry cell mass at 96 h respectively) when tryptophan and tryptophan in combination with leucine were added to the medium. Furthermore, surface culture and solid-state fermentation studies were also conducted in order to optimize the culture method for optimum CPT production. The highest CPT production of 3.37 ± 0.44 mg/100 g dry cell mass with cell mass of 22.79 ± 0.8 gl^{-1} was obtained with surface culture fermentation in shake flaks at 21 days of incubation time. The results obtained during solid-state fermentation on moist wheat bran and wheat bran supplemented with sabouraud broth indicated maximum productivity of 200.33 ± 19.90 µg/100 g dry weight and 390.78 ± 20.10 µg/100 g dry weight respectively at 21 days. The results of this study therefore suggest that surface culture may be advantageous to submerged fermentation for cultivation of *E. infrequens* and production of CPT. In addition to the optimization of the fermentation parameters in shake flasks, an attempt has been made to scale up the process in a bench scale bioreactor for the production of camptothecin under submerged conditions. The maximum yield of CPT represents a productivity of 0.575 ± 0.031 mg/100 g dry cell mass at 96 h in shake flasks whereas 4.96 ± 0.73 mg /100 g dry cell mass was recorded in 48 h in a bioreactor. CPT was identified by various physico-chemical tests coupled with spectroscopic analyses and further confirmed by its biological assay.

The literature scan indicate that there is no published report about any endophytic microorganism or independently growing microbes producing CPT. We have for the first time now reported the results of present study (Puri *et al.* 2005). In this study the possibility of CPT being produced by one or more microbes associated

with *N. foetidea* plants was also explored. The most logical place to focus the search for such microorganisms is one or more locations, which have naturally been supporting the growth of *N. foetida* for centuries. Thus, the indigenous isolates with the potential to produce novel bioactive molecules under a natural selection process could be utilized for developing alternate natural sources for these important compounds like CPT and its analogues.

The present work also describes the isolation of camptothecin and 9-methoxycamptothecin as marker compounds for LC-MS studies by semi-preparative HPLC. The purity of the isolates was determined by LC-MS and other spectral analysis. Earlier reported procedures for isolation of 9-methoxy-camptothecin from the crude extract involved cumbersome procedures encompassing high-speed counter current chromatography and repeated crystallizations. In the present methodology, 9-methoxy-camptothecin has been isolated to 95% purity by semi-preparative HPLC from the mother liquor after crystallization of camptothecin from the crude extract of *N. foetida.*

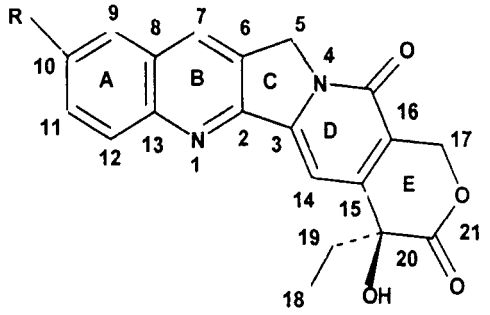
CPT is a promising antitumour alkaloid. From a practical viewpoint; microbial fermentation as a means of producing bioactive substance has several advantages like industrial production of a bioactive substance like CPT requires reproducible, dependable productivity. If a microbe is the source organism, it can be grown in tank fermenters as needed, generating a virtually inexhaustible supply of CPT. Enhancing productivity being relatively easy in microorganisms, it can be a reliable, convenient and economical source for inexhaustible supply of CPT and its derivatives.

The extension of the study for exploration of other secondary metabolites with this endophyte has lead me to other minor metabolites which may be useful to trace the secondary metabolic pathway of this organism. Besides two known secondary metabolites ergosterol and 5-(hydroxymethyl)-2-furfuraldehyde, interestingly the present studies have made possible to isolate three new complex molecules showing marked anticancer activity. These compounds have been characterized as substituted phenoxy caffeic acid and corresponding peroxy ferulic acid ester of polysubstituted salicylic acid (Fig a-f).

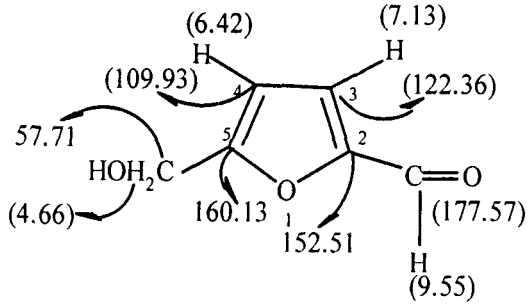
Admittedly, the present study is only indicative of alternate source of Camptothecin supply for the production of potent anticancer analogue. Extensive study would be required to further develop the isolated fungal strain as a potential organism for the development of economical fermentation process either in surface culture or in submerged deep tanks. Further more genomic and metablomic studies of the organism and the plant harbouring the organism would be interesting to know the secondary metabolite pathway used by the plant and microbes for generation of these important drug precursors.

Based on the HPLC, LC/MS, MS^2, ^{1}HNMR and ^{13}CNMR studies; structures of the six compounds isolated from the fungus have been characterized as shown (Fig. a-f) below.

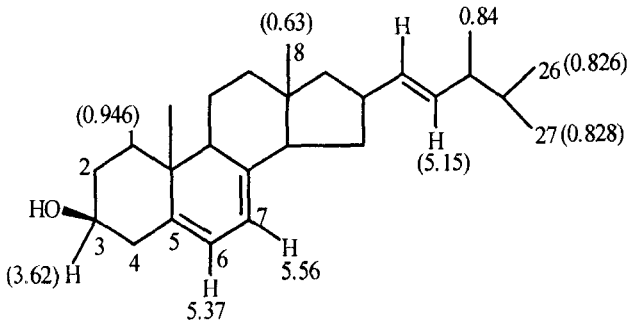
Known molecules



(a) Camptothecin, R=H

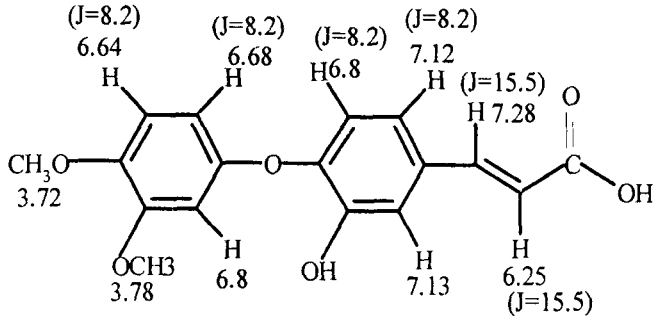


(b) 5-(hydroxymethyl)-2-furfuraldehyde

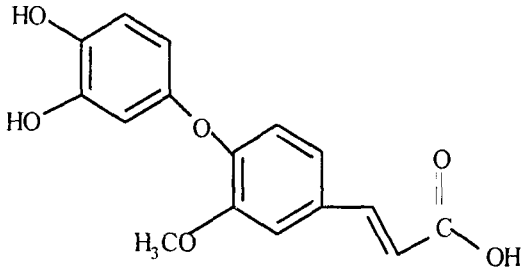


(c) Ergosterol

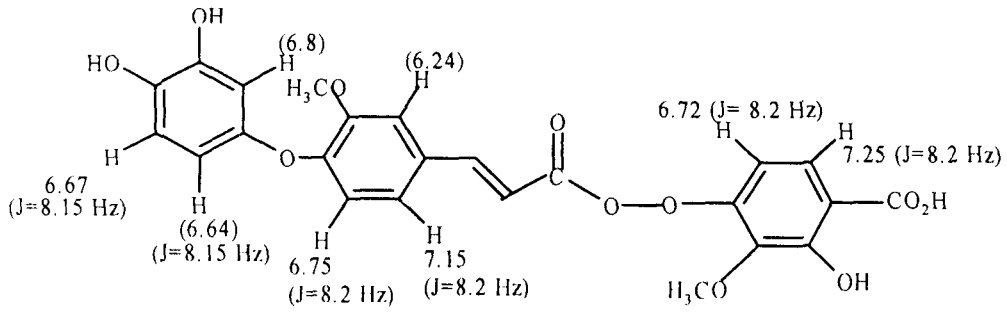
New molecules



(d) 4-(3, 4-dimethoxy-phenyl-1-oxy)-caffeic acid



(e) 4-(3,4-dihydroxy phenyl-1-oxy)-ferulic acid



(f) 4[4-(3,4-dihydroxyphenyl-oxy)-peroxy feruly]-3-methoxy-salicylic acid

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ANNEXURE

PATENT APPLICATION FILED

Puri, S. C.; Verma, V.; **Amna, T.**; Handa, G.; Gupta, V.; Verma, N.; Khajuria, R.K.; Saxena, A.K.; Qazi, G.N. and Spiteller, M. (2002). Camptothecin and Camptothecinoids from endophytic Fungi of *Nothapodytes foetida*. US patent. Docket No. 73663/JPW/MJW.

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PAPER PRESENTED IN CONFERENCES

Title: **Bioactive molecules from Endophytic microorganisms**

T. Amna, V. Verma, S.C. Puri and G.N. Qazi. Presented at ICOB-4 & ISCNP-24 IUPAC International Conference on Biodiversity and Natural Products: Chemistry and Medical Applications Held at New Delhi 26-31 January 2004.

Title: ***Invitro* cytotoxicity of camptothecinoids from *Entrophospora infrequens* against human cancer cell lines.**

T. Amna, G. Handa, A. Nazir, A.K. Saxena, S.C. Puri and G.N. Qazi. Presented as poster at CBISNF-2004 held at Vigyan Bhawan, New Delhi, Nov. 21-26, 2004. Poster No. 26-62.

Title: **Screening of different endophytes from *N. foetida* for anticancer compounds.**

A. Nazir, **T. Amna,** G. Handa, S.C. Puri and G.N. Qazi. Presented as poster at CBISNF-2004 held at Vigyan Bhawan, New Delhi, Nov. 21-26, 2004. Poster No. 26-9.

Title: **Quantification of Camptothecin in an Endophytic fungus by Liquid chromatography-Positive mode Electrospray Ionisation Tandem mass spectrometry (LC-MS/MS)**

T. Amna, R.K. Khajuria, S.C. Puri, V. Verma, J. Musarrat and G. N. Qazi. Presented at CRSI symposium, Indian Institute of Technology, Powai, Mumbai (accepted).

LECTURE DELIVERED

Title: **"Bioactive molecules from endophytic fungus *Entrophospora infrequens*"** at NCL, Pune on 8th to 10th November 2004.

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An Endophytic Fungus from *Nothapodytes foetida* that Produces Camptothecin

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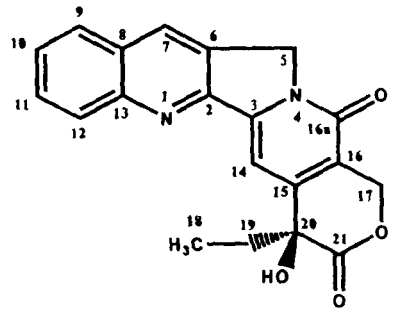
Abstract: A fungal endophytic isolate, camptothecin, has been isolated from the inner bark of the plant *Nothapodytes foetida* from the Western coast of India. The fungus, which belongs to the family Phycomycetes, produced the anticancer drug lead compound camptothecin (**1**) when grown in a synthetic liquid medium (Sabouraud broth) under shake flask and bench scale fermentation conditions. Compound **1** was identified by means of chromatographic and spectroscopic methods. It was also compared with an authentic example for its biological activity against a number of human cancer cell lines. Isolation of an organism producing **1** and its fermentation may, in the future, provide an easily accessible source for the production of this anticancer drug precursor molecule.

Camptothecin (**1**), a pentacyclic quinoline alkaloid, belongs to a group of antineoplastic agents with a unique mechanism of action involving interference with eukaryotic DNA.[1-4] Moreover, one of the primary cellular responses to its exposure is a rapid cessation of RNA synthesis.[5] This alkaloid displays a unique mechanism of action by inhibiting the intranuclear enzyme topoisomerase I, which is required for the swivelling and relaxation of DNA during molecular events, such as DNA replication and transcription.[6]



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20(*S*)-Camptothecin, the naturally occurring enantiomer, was first isolated by Wall et al.[7] from the wood of *Camptotheca acuminata* Decne (Nyssaceae), which is a plant native to mainland China. Camptothecin and its derivatives show strong antineoplastic activity. The drug is already used in China for the treatment of skin diseases.[8] Hycamtin (topotecan) and Camptosar (irinotecan), semisynthetic derivatives of **1**, have been employed clinically for the treatment of ovarian and colon cancers.[9,10] Compound **1** is also used as an insect chemosterilant, a plant regulator, and an inhibitor of the herpes virus.[11] In addition, compound **1** prevents the replication of the influenza virus.[12] Compound **1** and minor camptothecinoids have been obtained in high yield from the Indian tree *Nothapodytes foetida* (Wight) Sleumer (formerly *Mappia foetida*, Icacinaceae),[13-15] commonly known in India as "Kalgur". This small tree is distributed in the western part of peninsular coastal India from Konkan ghats to northern parts of the Kanara, Niligiris, Anamalis, and Pullneys hills. Compound **1** has also been reported to be present in various Japanese plant species, including *N. collina*, *N. obscura*, *N. obtusifolia*, *N. piltosporsides*, and *N. tomentosa*.[16]

Camptothecin (**1**) is not abundant and is only available in relatively low concentrations in the roots of *Nothapodytes* species, which unfortunately demands the uprooting of rare, 50- to 75-year-old trees from the forests. The supplies of **1** available from inconsistent wild sources are, therefore, inadequate when compared to the projected demand. While a synthetic route to **1** has been reported, the yield, after a multistep procedure, is low, commercially insignificant, and

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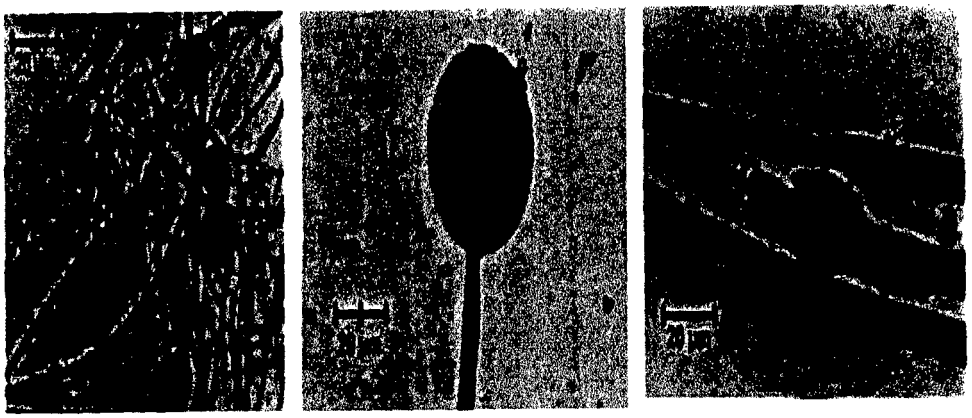

Figure 1. (a) Microscopic view of horizontally growing unbranched stoloniferous hyphae (×500). (b) Microscopic view of the young sporangium of endophytic fungus (×1000). (c) Microscopic view of horizontally growing unbranched stoloniferous hyphae (×1000).

hence nonviable.[17-19] Therefore, it is essential to find alternative sources of **1** to meet the pharmaceutical demand.

Herein we report, for the first time, the production of the quinoline alkaloid camptothecin by an endophytic fungus (RJMEF001) isolated from *N. foetida* (Figure 1a–c), a plant from Konkan ghats in India and presently being maintained in the botanical gardens of Regional Research Laboratory, Jammu. Molecular analysis of the fungus based on a large subunit (LSU) ribosomal RNA gene revealed 99.8% similarity to *Entrophospora infrequens* and also to other related taxa, e.g., *Rhizopus oryzae* strains UWFP 973 and 846 (98.6%). Further investigations of this nature are currently underway. The fungal strain has been deposited at MTCC, Chandigarh, India (MTCC 5124), and a PCT application has also been filed.[20]

A literature survey shows that the antitumor diterpenoid paclitaxel and its congeners have been reported as being produced from an endophytic fungus called *Taxomyces andreanae*.[21-22] There is no published report that **1** might be produced by any microorganism associated with a plant species or growing independently in nature.

The identity of **1** in the fungal isolates was confirmed by chromatographic and analytical methods, such as optical rotation and UV, IR, CD, LC/MS, LC-MS/MS, HRMS, and ^{1}H and ^{13}C NMR spectra. The quantitative estimation of **1** by HPLC against standard **1** (derived from plant) indicated a yield of 18 μg/mg of the chloroform extract after 6–7 days of incubation of the isolated microorganism under shake flask conditions. The electron impact mass spectrum (EIMS) of fungal camptothecin (**1**) was identical (Figure 2) to the published spectrum[23] of this molecule from a plant source, having a molecular ion peak at *m/z* 348 with characteristic fragments at *m/z* 319 (M − ethyl), *m/z* 304 (M − CO_2), *m/z* 291 (*m/z* 319 − CO), *m/z* 290 (*m/z* 319 − CHO), *m/z* 275 (*m/z* 304 − ethyl), *m/z* 248 (*m/z* 275 − HCN), and *m/z* 247 (*m/z* 275 − CO). In the ESIMS, the molecular ion of camptothecin exhibited *m/z* 349 $(M + H)^+$, and in the MS/MS mode, characteristic fragment peaks (Figure 2) could be observed.

The biological activity of fungal **1** was tested using an in vitro cytotoxicity assay[24] against human cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) in comparison with the standard **1**, resulting in comparable activities.

Optimization of fermentation conditions for the efficient production of **1** by fermentation is underway. This may lead to the development of an economical and eco-friendly process for the production of camptothecin (**1**) by fermentation to meet the ever-increasing demand for the compound as a unique anticancer drug precursor molecule.

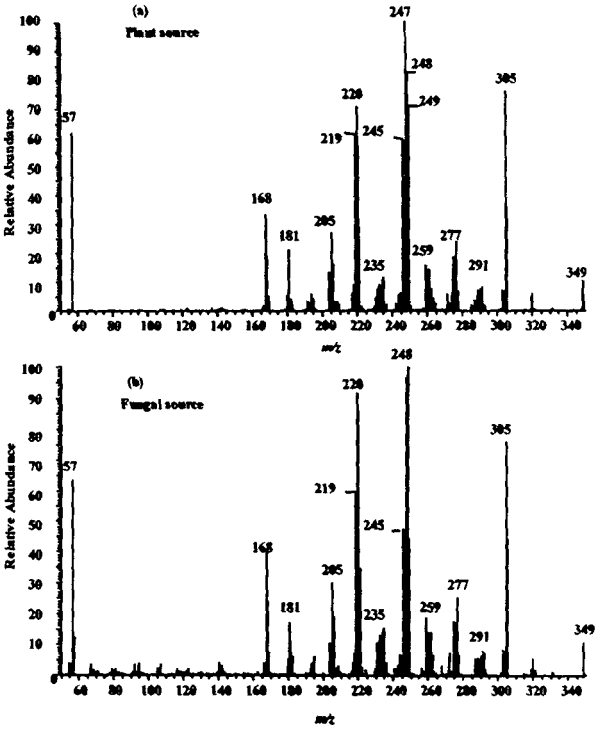

Figure 2. ESIMS/MS of **1** from (a) plant source and (b) fungal source.

Experimental Section

General Experimental Procedures. Low-resolution MS was performed by EI ionization (Finnigan-MAT 8000) at 70 eV, with a direct inlet probe at 252 °C. A Finnigan TSQ 7000 with ESI ionization in the MS/MS mode was used. The optimal collision energy (Figures 2a,b) was determined by means of an ICL procedure controlling the automatic switching between different voltages, with a step size of 0.5 V/scan to 40 V. During this procedure, the analytes were injected via a Rheodyne valve with a 2 μL injection loop at a concentration of 10 μg mL^{-1}. A prescan voltage settling time of 20 min and 0.4 s for one complete cycle (four transitions) was used for selected reaction monitoring (SRM). HRMS was done using a JEOL JMS/SX 102 A FAB ion source (matrix, 3-nitrobenzyl alcohol; calibration, PEG 400; resolution, 10 000) and an Apex III FTICR mass spectrometer (Bruker Daltonics, Billerica, MA) with a 7 T superconducting magnet. Positive ions were

produced in an external Apollo electrospray ion source (Bruker Daltonics, Billerica, MA) with a flow rate of 2 mL min^{-1}. Infrared multiphoton dissociation (IRMPD) and activation of ions in the ICR trap were performed using a CO_2 J48-2 laser with 25 W maximum power output (Synrad, Mukilteo, WA). The optical rotation measurement was performed using a Perkin-Elmer 341 polarimeter with a tube of 10 cm cell path length using $CHCl_3$–MeOH (8:2) as solvent. CD was performed using a JASCO J715 spectropolarimeter, with a Hellma precision quartz glass Suprasil cuvette, a 1 mm light path length, and $CHCl_3$–MeOH (4:1) as solvent. FTIR Bruker IFS (KBr) was used for recording the IR spectra. UV spectra were obtained using a Varian CARY 100 BIO, 1 cm cuvette, and $CHCl_3$–MeOH (4:1) as solvent. For ^1H and ^{13}C NMR spectra a Bruker AMX 600 instrument was used.

Isolation of Endophytic Fungi. Fresh plant material was taken, and small stems explanted from the fully matured *Nothapodyte foetida* tree (containing a measurable concentration of **1**) were treated with 95% ethanol as a disinfectant. Pieces of the inner bark of the stem were placed on aqueous agar (AG) and incubated at 28 ± 2 °C until fungal growth started. The tips of the fungal hyphae were removed from the AG and placed on a rich synthetic mycological medium (e.g., Sabouraud agar, SBA, containing dextrose 4%, peptone 1%, and agar 2%). The pure culture, thus obtained, was preserved by lyophilization, as well as by cryopreservation at −70 °C. The fungus grows as a white cottony mycelium when young. The well-developed mycelium is branched, fast growing, and spreads on the solid medium. Aerial hyphae are produced after growth for 5–7 days and turn black due to sporulation. Microscopic slides were prepared by following standard methods.[25]

Isolation of Total Genomic DNA. Total DNA was isolated from the mycelial mass using the standard method[26] with slight modifications. The DNA was resuspended in a suitable volume of TE buffer (10 mmol of Tris-HCl, pH 8.0, 1 mmol, EDTA). DNA was quantified spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany).

Identification of the Isolate. The fungus was identified using a Microseq D2, large subunit (LSU) rDNA fungal sequencing kit ABI (Applied Biosystems, Foster City, CA). The LSU ribosomal gene (~300 bp) was amplified and sequenced on an ABI Prism 310 genetic analyzer (ABI, Foster City, CA). The DNA sequences thus obtained were submitted to the ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) and the sequences aligned to identify the fungus.

Preparation of Cell-Free Extract and Chromatographic Separation. The cell-free extract was prepared by filtering the incubated culture grown in Sabouraud broth (dextrose 4%, peptone 1%) through muslin cloth, resuspending the mycelial pellet in deionized water, and sonicating the mixture in a Branson sonifier. The milky fluid was extracted three times with an equal volume of $CHCl_3$–MeOH (4:1), after which the organic solvent was removed by rotary evaporation at 30 °C, yielding the organic extract. HPLC separation was performed using a Luna RP-18 column (2 mm i.d., length 150 mm, particle size 3μm) and a guard column (Phenomenex, Torrance, CA) at a flow rate of 200 μL min^{-1} at 30 °C. A 10 μL amount of sample was injected in $CHCl_3$–MeOH (4:1). The mobile phases water (A) and acetonitrile (B) were changed in the following manner: 0–5 min 90% A and 10% B, 5–20 min 40% A and 60% B, 20–30 min 2% A and 98% B, 30–32 min 2% A and 98% B, and 35–37 min 90% A and 10% B v/v. The UV signal was recorded at $\lambda = 256$ nm. The retention time of **1** was 20.15 min.

In Vitro Cytotoxicity against Human Cancer Cell Lines. Three selected cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) were grown for 24 h on 96-well tissue culture plates. Incubation was continued for another 48 h after addition of the test material dissolved in DMSO (final concentration of DMSO <1%) into each well except for the wells that acted as a control or wherein a known drug was added. Cell growth was terminated by addition of trichloroacetic acid. Cells were stained with sulforhodamine B (SRB). Excess dye was removed by washing with water. The bound dye was dissolved in tris-buffer and read using ELISA.

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Supporting Information Available: Conditions of fungal growth and physical and spectroscopic data for camptothecin (**1**) obtained. This information is available free of charge over the Internet at http://pubs.acs.org.

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Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin

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Abstract: Twigs (young and old) from *Nothapodytes foetida* growing in the Jammu and Mahabaleshwar regions in India were used for the isolation of 52 strains of endophytic fungi and were tested for their ability to produce the anticancer alkaloid camptothecin. One of the isolates from the inner bark tissue of the *N. foetida* plant growing in the Jammu region of J&K state, India, was found to produce detectable quantities of camptothecin and its derivatives when grown in a semi-synthetic liquid medium. Camptothecin was identified by physicochemical analysis and further confirmed by spectroscopic studies. No camptothecin was detected in zero time cultures or in uninoculated culture broth. The maximum yield of camptothecin was 0.575 ± 0.031 mg/100 g of dry cell mass in 96 h in shake flasks, whereas 4.96 ± 0.73 mg/100 g of dry mass was recorded in 48 h in a bioreactor.

Key words: endophytes, camptothecin, anticancer drug, *Nothapodytes foetida*, *Entrophospora infrequens*, bioreactor, scale-up.

Résumé : Des brindilles (jeunes et vieilles) de *Nothapodytes foetida* poussant dans la région de Jammu et Mahabaleshwar en Inde ont été soumises à l'isolation de 52 souches de champignons endophytes et ont été analysées pour leur capacité à produire un alcaloïde anticancéreux, la camptothécine. L'un des isolats issus d'un tissu de l'écorce intérieure de la plante *N. foetida* poussant dans la région de Jammu dans l'état de J&K, Inde, a produit des quantités détectables de camptothécine et de ses dérivés lorsque cultivée dans un milieu liquide semi-synthétique. La camptothécine fut identifiée par analyse physico-chimique et confirmée par la suite par spectroscopie. Aucune camptothécine ne fut détectée dans des cultures au temps zéro ni dans le bouillon de culture non inoculé. Le rendement maximal de camptothécine représente une productivité de 0,575 ± 0,031 mg/100 g de masse cellulaire en 96 h dans des flacons à agitation alors que 4,96 ± 0,73 mg/100 g de masse sec fut mesuré en 48 h dans un bioréacteur.

Mots clés : endophytes, camptothécine, médicament anticancéreux, *Nothapodytes foetida*, *Entrophospora infrequens*, bioréacteur, extrapolation.

[Traduit par la Rédaction]

Introduction

It has been estimated that there may be as many as 1 million different fungal species on our planet (Hawksworth and Rossman 1987). In the past century, many of the 0.1 million fungi that have been described have been found associated with various higher organisms as either parasites or saprophytes. The habitats of the remaining 0.9 million fungi are unclear. Microorganisms seem to occupy virtually every living and nonliving niche on earth, including thermal vents, deep rock sediments, and desert and marine environments. In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of organisms known as endophytes (Bacon and White 2000). Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots, and leaves of plants. They cause no discernible manifestation of their presence and have typically gone unnoticed (Strobel and Long 1998). As a result of these long-held associations, it is conceivable that some of these endophytic microbes may have devised genetic systems allowing for the transfer of information between themselves and the higher plant and vice versa (Stierle et al. 1993).

The symbiosis between plant and endophyte has been ascertained: the former protects and feeds the latter, the latter in turn produces bioactive substances (plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal substances) to enhance the growth and competitiveness of the host in nature (Carroll 1988). A worldwide scientific effort to isolate endophytes and study their secondary metabolism is now under way (University of Wales 2001). In fact, a re-

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cent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown (Schutz 2001).

In the present study, it was observed that an endophytic fungus isolated from the inner bark of *Nothapodytes foetida* growing in the Jammu region of India was able to synthesize camptothecin (CPT). CPT, which was first isolated from *Camptotheca acuminate* Decne (Nyssaceae) (Wall et al. 1966), is an important chemotherapeutic agent with unusual efficacy against lung, ovarian, and uterine cancers (Yakugyo 1995). CPT and its analogues act as strong inhibitors of topoisomerase 1 by trapping the cleavable DNA – topoisomerase 1 complex (Potmesil and Pinedo 1995; Sawada et al. 1995; Torck and Pinkas 1996). CPT is found naturally in several varieties of plants, including *C. acuminatea* (Nyssaceae) (Wall et al. 1966), *N. foetida* (Wight) Sleum (Icacinaceae) (Govindachari and Viswanathan 1972), *Merilliodendron megacarum* (Helmsl) Sleum (Icacinaceae) (Arisawa et al. 1981), *Ervatomia heyneana* (Wall et al. 1966) T. Cooke (Aocynaceae) (Gunasekera et al. 1990), and *Ophiorriza mungos* Linn (Rubiaceae) (Tafur et al. 1976).

Although CPT is a promising anti-tumor agent, it is unfortunately only available in relatively insufficient concentrations in the tree roots, which demands the uprooting of rare 50- to 75-year-old trees. The supplies of CPT available from inconsistent wild sources are therefore inadequate when compared with its projected demand. Although the synthesis route for CPT has been reported, the yield after the multistep procedure is low and, therefore, of no commercial significance (Wall et al. 1993; Rao et al. 1994; Fang et al. 1994). Thus, it is essential to completely understand the biosynthesis of CPT in the plants and to evaluate the factors that affect biosynthesis. Additionally, it is important to search for alternative natural sources of this important compound. This paper deals with the isolation of an endophyte from *N. foetida* plant, the optimization of fermentation parameters in the shake flasks, and the scale-up in a bioreactor for the production of CPT.

Materials and methods

Isolation of endophytic fungi

Fresh plant material was taken and small stems explanted from fully matured *N. foetida* trees. The material was treated with 95% ethanol, and pieces of the inner bark of the stem were placed on aqueous agar and incubated at 28 ± 2 °C until fungal growth started. The tips of the fungal hyphae were then removed from the aqueous agar and placed on mycological medium (i.e., potato dextrose agar (diced potatoes, 300 g/L; dextrose, 20 g/L; agar, 20 g/L) or Sabouraud agar (dextrose, 40 g/L; peptone, 10 g/L; agar, 20 g/L)). The pure cultures obtained were transferred to a number of solid and liquid media that support fungal growth (viz., potato dextrose agar (diced potato extract, 300 g/L; dextrose, 20 g/L; agar, 20 g/L), malt extract agar (malt extract, 30 g/L; peptone, 5 g/L; agar, 20 g/L), or Sabouraud agar (dextrose, 40 g/L; peptone, 10 g/L; agar, 20 g/L)). In this process, we were able to isolate 52 different endophytic fungi. Each microbe was grown in liquid Sabouraud medium (100 mL) and screened for CPT production. One isolate indicating the presence of CPT was selected for further investigation. The endophytic fungus grew as white, cottony mycelia in its early growth phase, followed by well-developed branched mycelia on the solid Sabouraud medium. Aerial hyphae were produced after 10–12 days of growth; they turned black because of sporulation. Microscopic slides were prepared, stained by Lectophenol cotton blue, and examined using an Olympus light microscope (Bionocular Olympus OIC, make IEBI, USA).

Geographical occurrence

The fungus was found in plant samples of *N. foetida* (the plant originated in the Western Ghats and was later introduced to the temperate Jammu region of India). Several fungal strains were isolated from the explants of these trees. Following serial dilution, a pure axenic culture was obtained that was later identified as *Entrophospora infrequens*, using standard identification protocols (http://rdp.cme.msu.edu and http://ncbi.nlm.nih.gov).

Maintenance media

The culture of the isolated endophyte *E. infrequens* was routinely maintained on Sabouraud agar containing glucose (40 g/L), peptone (10 g/L), and agar (20 g/L), pH 5.5.

Sporulation

The specific sporulation medium described by Khajuria et al. (2001) was used to generate profuse sporulation. Molasses medium prepared in Roux bottles and autoclaved at 121 °C for 15 min was used to the produce spores. The medium was inoculated with a spore suspension and incubated at 28 ± 2 °C. Spores were harvested from solid culture (see below) after 8 days of incubation, when profuse sporulation was observed.

Harvesting of spores

The spores were harvested using Tween-80 (0.01% *v/v* aqueous solution) to dislodge them out of the mycelium mass. The suspension was suctioned filtered through a Whatman filter paper No. 1 using a Millipore assembly (Millipore Corp., Bedford, Massachusetts, USA) and filtrates were pooled and, after appropriate dilution, the spores were counted using a hemocytometer (Counting Chamber, Nuebaur, Germany).

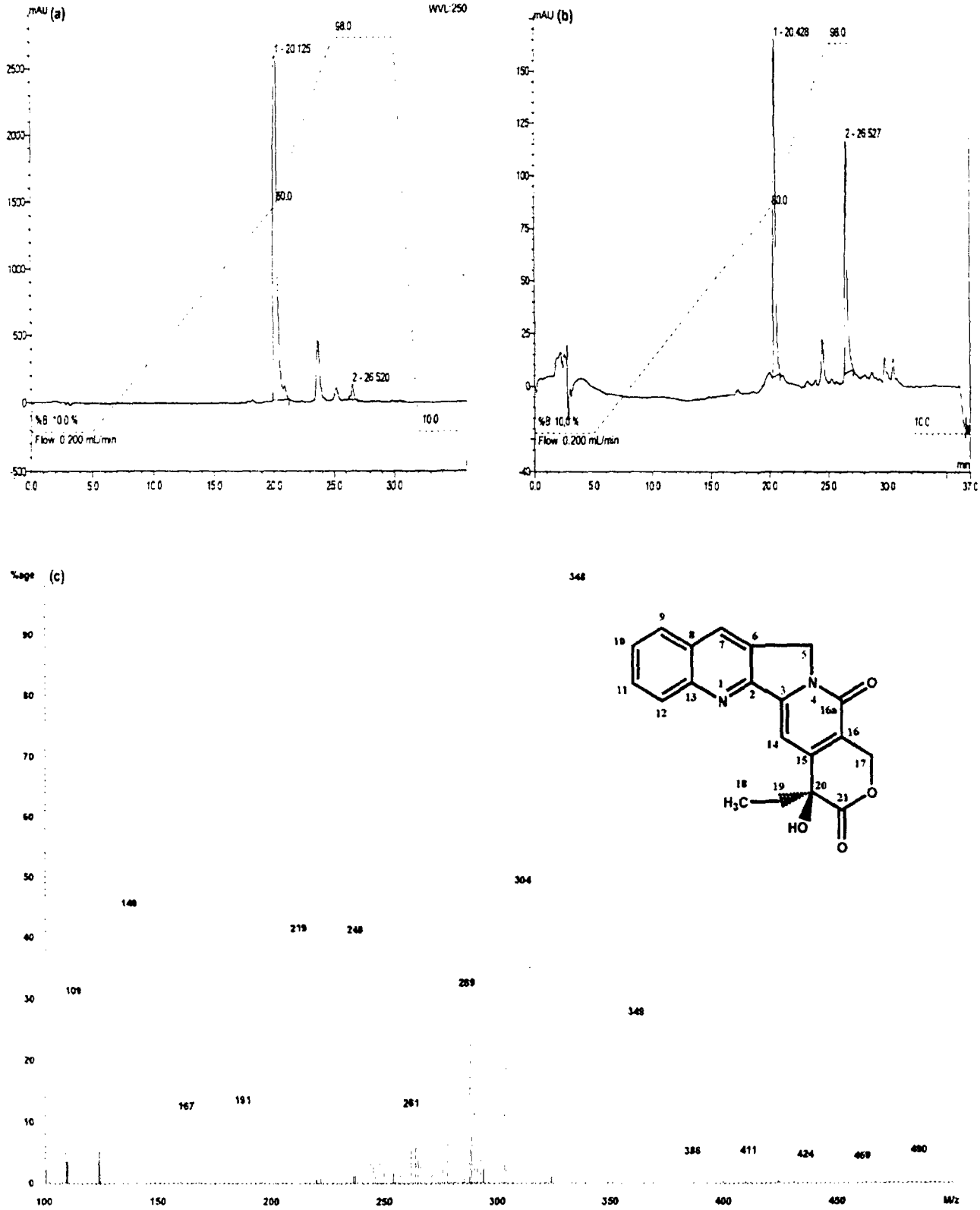
Scanning electron microscopy

Recovered spores were fixed and processed using the modified method of Millonig (1961). The spores were recovered on a clean coverslip and fixed in 2.5% (*v/v*) glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 2 h. The material was postfixed in 1% (*v/v*) osmium tetraoxide in the same buffer for 3 h, dehydrated in a graded ascending acetone gradient (10%–100%), and dried using carbon dioxide. The samples were then mounted on stubs, coated with carbon in a JEOL-JEE4X vacuum evaporator, and then coated with gold in a Polaron sputter coater. Finally, the samples were observed in a JEOL-100CXII electron microscope with ASID operating at 40 kV.

Growth of organism

The organism used in the present study was grown in presterilized Sabouraud broth with dextrose monohydrate (4% *m/v*) and peptone (1% *m/v*) as the sole carbon and nitrogen sources, respectively. Dextrose monohydrate was autoclaved

Fig. 1. Liquid chromatography profile of (*a*) authentic camptothecin, (*b*) fungal camptothecin, and (*c*) mass of fungal camptothecin.



separately at 115 °C for 10 min. Shake-flask experiments were carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium, with agitation at 200 r/min (1 r = 2π rad) on a rotary shaker at 28 ± 2 °C. Large-scale cultivation of fungus was performed in a 40 L bioreactor (New Brunswick Scientific, Edison, New Jersey, USA). The working volume was kept at 18 L with an aeration rate of 1 vvm, a vessel pressure of 0.2 kg/cm, a temperature of 28 ± 2 °C, and an

Fig. 2. Microscopic view of (*a*) horizontally growing unbranched stoloniferous hyphae (× 500), (*b*) horizontally growing unbranched stoloniferous hyphae (× 1000), and (*c*) the young sporangium of endophytic fungus (× 1000).

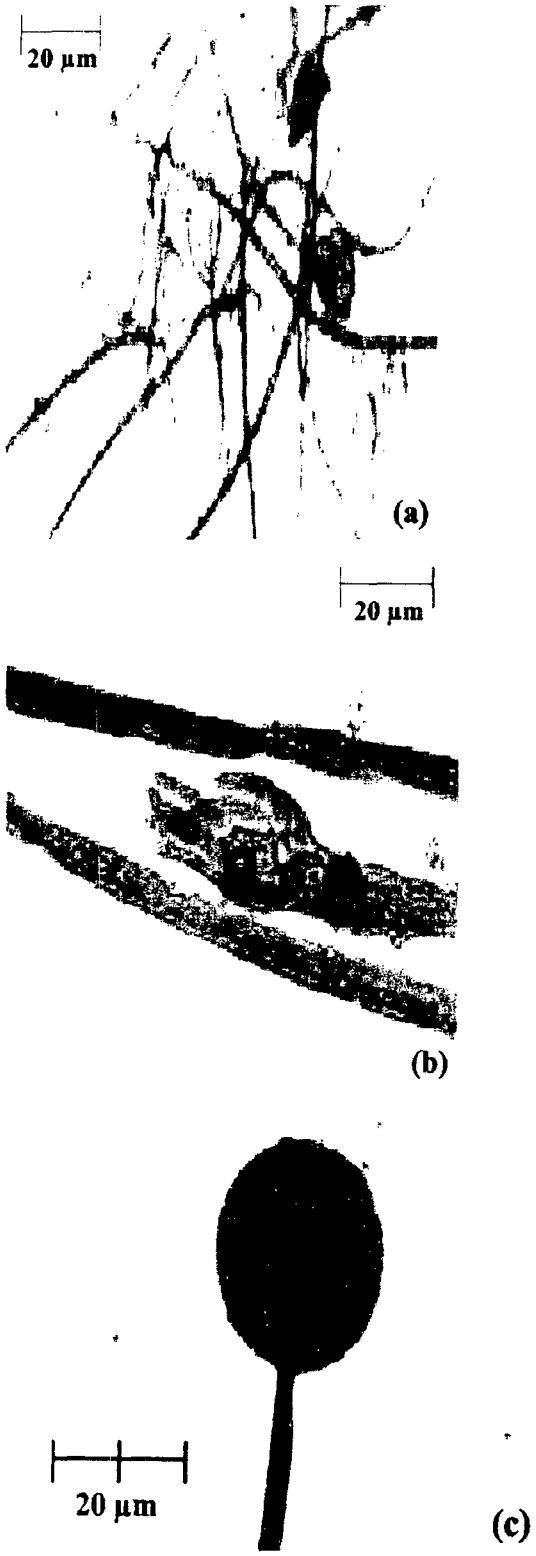
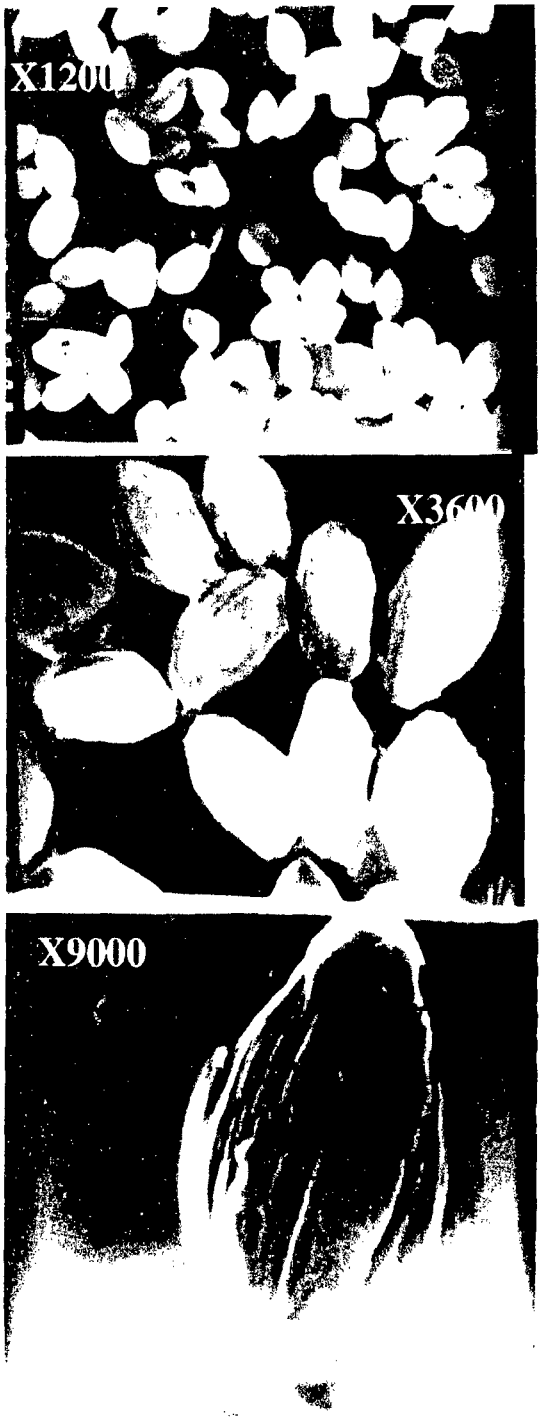
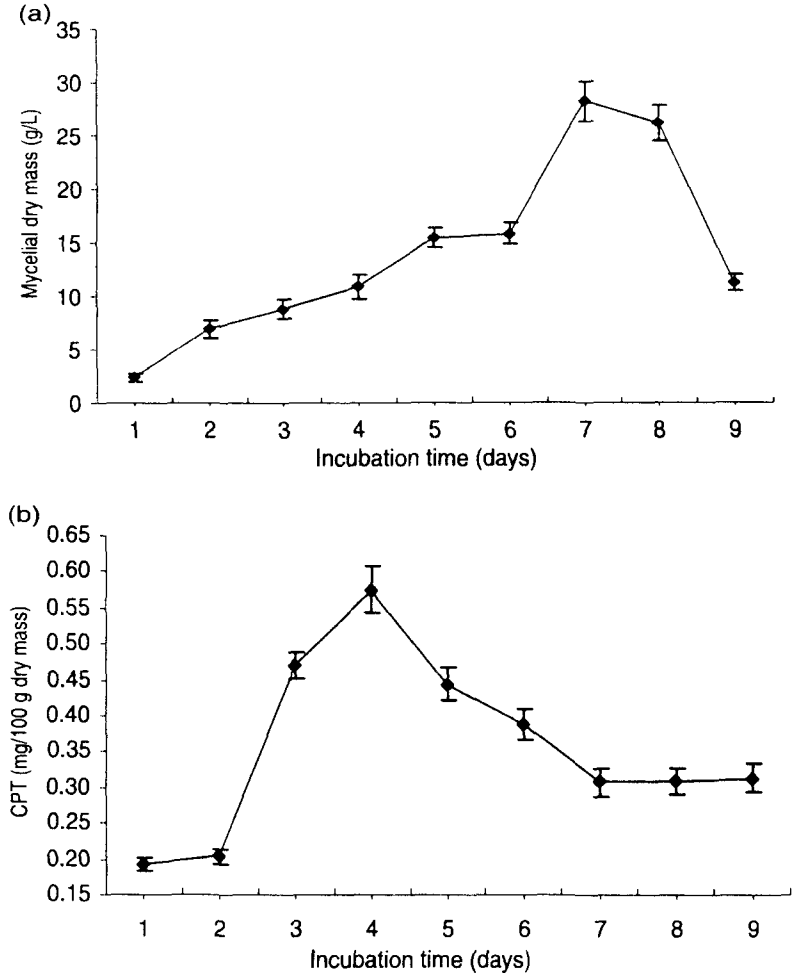


Fig. 3. Scanning electron micrographs depicting detailed surface ultrastructural characteristics of spores of *Entrophospora infrequens*.



agitation rate of 220 r/min. In all the experiments, the initial spore count in the broth was 1×10^5 spores/mL, and samples were drawn 2 h after inoculation (0 h) and every 24 h thereafter until either 216 h (for shake-flask experiments) or 120 h (for bioreactor experiments). Residual sugar, pH, dissolved oxygen, and CPT production were determined at each time point.

Fig. 4. (*a*) Growth profile of *Entrophospora infrequens*. (*b*) Production profile of *E. infrequens* in shake flask. Data represent the mean of three replicates ± SD, and point-to-point comparison indicates biomass production at day 7 and camptothecin (CPT) production at day 4. Statistical significance was determined by the unpaired Student's *t*-test.



The aeration rate was controlled by a mass flow controller (Brooks Instruments, Hatfield, Pennsylvania, USA). Prior to inoculation, the air flow rate was fixed at 1 vvm, which was maintained until the end of the culture period. Under these conditions, an adequate mixing of the spore suspension (seed) was achieved throughout the culture period. Silicone oil (Himedia Laboratories, Mumbai, India) was added to avoid foam formation.

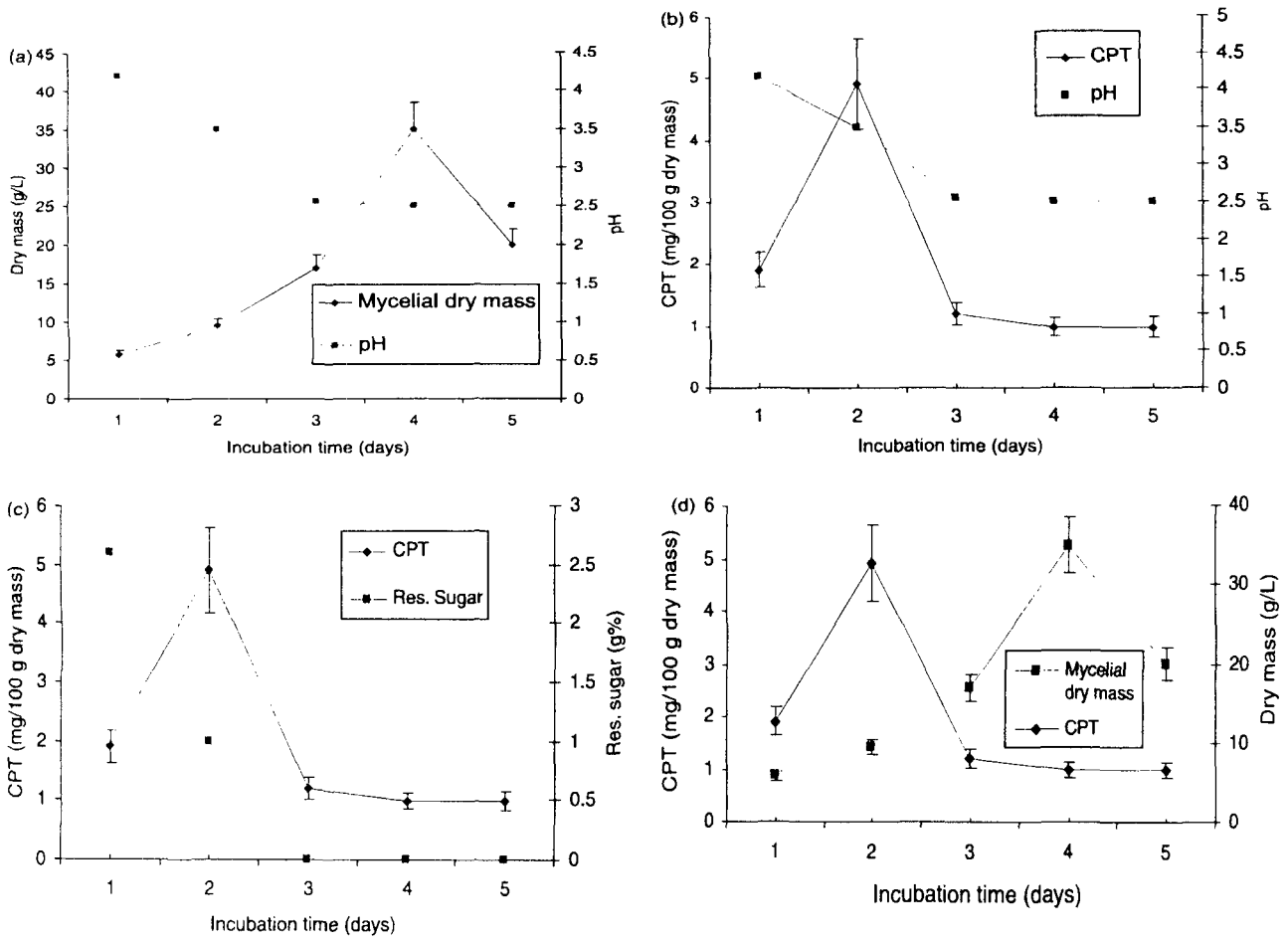
The wet mass of the mycelium pellet obtained after suction filtration of a known volume of fungal culture was recorded. The mycelium pellet was then washed with several volumes of distilled water to remove the residual medium. A representative sample of pellet was dried at 50 ± 2 °C for 14–18 h and the dry mass per litre was calculated.

Sugar estimation was made according to an established potassium ferricynide method (Coles 1942).

Extraction and CPT measurements

CPT was extracted from the fungal mycelia as described by Yan et al. (2003). Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile distilled water and homogenized in a cell disintegrator. The cell homogenate was extracted four times with equal volumes of laboratory-reagent-grade chloroform–methanol (4:1 *v/v*) mixture. Solvent was distilled off in a rotary evaporator, leaving behind organic residue. The residue was applied to silica gel TLC plates (Merck K GaA, 64271 Darmstadt, Germany) run in a (9:1 *v/v*) chloroform–methanol solvent system. The spots that developed were superimposable with the CPT standard. The spots were detected under ultraviolet (UV) illumination at λ_{max} 256 nm. A compound having chromatographic properties similar to CPT was identified by comparative TLC, high pressure liquid chromatography (HPLC) (Figs. 1*a*–1*c*) when the separation was performed using a Luna RP18 column (2 mm internal diameter, 150 mm length, 3 μm particle size), and a safety guard (Phenomenex, Torrance, California, USA) at a flow rate of 200 μL/min at 30 °C. Ten microlitres of sample was injected in a $CHCl_3$–MeOH (4:1, *v/v*) solvent. The mobile phase consisting of water (A) and acetonitrile (B) was run in the gradient as follows: (0–5 min 90% A, 10% B; 5–20 min 40% A, 60% B; 20–30 min 2% A, 98% B; 30–32 min 2% A, 98% B; and 36–37 min 90% A, 10% B; *v/v*). The UV signal was recorded at λ_{max} 256 nm. Retention time of CPT was 20.125 min, and the compound was finally characterized by liquid chromatography – mass spectrometry (LC–MS) and

Fig. 5. Effect of incubation period on (*a*) biomass and pH, (*b*) camptothecin (CPT) and pH, (*c*) CPT and residual sugar, (*d*) biomass and CPT production by *Entrophospora infrequens* in the 18 L bioreactor. Data represent the mean of three replicates ± SD and point-to-point comparison indicates biomass production at day 4 and CPT production at day 2. Statistical significance was determined by the unpaired Student's *t*-test.



LC–MS/MS, high resolution MS, and ^{1}H and ^{13}C NMR spectra. CPT production was followed as a function of incubation time in shake-flask and bioreactor cultures. To quantify the CPT in each extract, the quantitative method described by Palumbo et al. (2001) was followed.

Results and discussion

Organism characteristics

Entrophospora infrequens is reported here as an endophytic fungus associated with the inner bark of *N. foetida*. We observed that the fungus grew on many common media in 10–12 days. The fungus has small hyphae that average 2–3 μm in diameter (Figs. 2*a* and 2*b*). The mycelia are branched and aseptate, ribbon shaped, and multinucleate. Sporangiophores are long, unbranched, wide, and terminate in a sporangium (Fig. 2*c*). The sporangium is oval and bears round to oval spores. The outer surface of spores bears prominent striations (Fig. 3). The fungus was deposited at the International Culture Repository MTCC Chandigarh, India under accession No. 5124.

Shake-flask experiments for the production of CPT

Shake-flask experiments with the fungal culture were conducted to evaluate the optimal conditions for growth and its capacity to produce secondary metabolites. Data represent the mean of three replicates and the experiment was repeated six times to get reproducible results. The inoculation conditions and the fungal culture in 500 mL Erlenmeyer flasks are summarized in Table 1. In shake flasks, the growth period of the culture extended to 6 days following incubation and peaked on day 7 (Fig. 4*a*). No sporulation was observed in submerged cultures. The fungal mass of 2.30 ± 0.43 g/L was measured at 24 h and the maximum production of 28.11 ± 1.92 g/L was measured on day 7. CPT accumulation in the cell mass was evident by 24 h and continued through to 96 h (Fig. 4*b*); thereafter, a decline in the content was observed. The highest cell-associated CPT content was found to be 0.575 ± 0.031 mg/100 g dry mass of mycelia. Production of the secondary metabolite (CPT) was found to precede growth in the shake-flask experiments. The spectrometric data of pure fungal CPT was obtained by repeated column chromatography of organic extract as pale yellow amorphous pow-

Table 1. Cultivation parameters for *Entrophospora infrequens*.

Parameter	Shake flask	Bioreactor (18 L)
Inoculum	Spores (10^5 spores/mL)	Spores (10^5 spores/mL)
Medium	Sabouraud	Sabouraud
Cultivation time	216 h	120 h
Fermentation modus	Batch	Batch
Working volume	100 mL	5–18 L
Temperature	28±2 °C	28±2 °C
Vessel pressure	—	2 lbs
Aeration rate	—	1 vvm
Antifoam	—	Silicon oil
Agitation (r/min)	200–220	200–220

der ($CHCl_3$–MeOH). $[\alpha]_D^{30}$ +29.6° (*c* 0.04, $CHCl_3$–MeOH, 4:1). UV ($CHCl_3$–MeOH, 4:1) λ max (intens., nm): 256 (0.800), 290 (0.167), 371 (sh, 0.511); ε_{361} = 18.300; CD ($CHCl_3$–MeOH, 4:1) λ max (Δ ε): 295 (4) 214 (-6), 220, 219 (2), 237 (36), 257 (sh, 10). IR (KBr, cm^{-1}) ν_{max}: 1751, 1741, 1652, 1602, 1580, 1500, 1438, 1349, 1324, 1252, 1234, 1197, 1157. ^1H NMR ($CDCl_3$, 200 MHz, *J* in Hz) δ: 0.90 (1H, t, *J* = 7.2, H-19), 1.88 (1H, q, H-19), 5.27 (2H, s, H-5), 5.44 (2H, s, H-17), 7.31 (1H, t, *J* = 8.5, H-11), 7.36 (1H, s, H-13), 7.71 (1H, t, *J* = 8.5, H-10), 8.12 (1H, d, *J* = 8.5, H-9), 8.17 (1H, d, *J* = 8.5), 8.68 (1H, s, H-7). ^{13}C NMR (DMSO, 151 MHz) δ: 152.5 (C2), 145 (C3), 50.2 (C5), 129.8 (C6), 131.5 (C7), 128.5 (C8), 127.6 (C9), 127.9 (C10), 147.9 (C13), 96.7 (C14), 149 (C15), 119.0 (C16), 156.8 (C16a), 65.2 (C17), 7.7 (C18), 30.3 (C19), 72.3 (C20). EI-MS *m/z*: 348 $(M)^+$ (100), 304 (44), 289 (28), 261 (69), 248 (35), 219 (36), 191 (9), 140 (2), 105(7). ESI-MS/MS *m/z*: 320 $[M + H–ethyl]^+$, 305 $[M + H\text{-}CO_2]^+$, 277 $[M\text{-}CO_2 + CO]^+$, 249 [M + H-100, CO_2 + CO + ethyl]$^+$, 248 [M + H-101, M-72, and ethyl]$^+$, 220 [M-128, M-72-ethylene–CO]$^+$. HR-ESI-IR – MPD-MS/MS *m/z*: 349.11925, 305.12930, 277.13429, 249.10288. FAB-MS *m/z*: calcd. for $C_{20}H_{17}N_2O_4$: 349.1176; found: 349.1147 $[M+H]^+$.

Growth and CPT production in bioreactors

Different batches of bioreactors from 5 L (Airlift Lh, Model 5-2000 series, UK) to 18 L (NBS, New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) capacity were run for the optimization of fermentation conditions for the maximum growth of biomass and production of CPT. These bioreactors were run under similar and simulated conditions and also under different conditions for optimization of the fermentation parameters for maximum CPT production. The conditions found to be optimal for the growth of *E. infrequens* and production of secondary metabolite are summarized in Table 1. Growth of mycelium was observed to begin 2–3 h after inoculation. The growth peaked at 96 h in the 18 L bioreactor, after which a gradual fall in biomass production was observed, which might be owing to partial lysis of cells after attaining stationary phase. A temperature of 28 ± 2 °C was found to be optimal for the growth of mycelium and CPT production. The pH of the medium in the bioreactor was initially recorded to be 5.6; the pH decreased upon growth, reaching 2.5 at 120 h of fermentation. At the start of the stationary phase (96 h), 35.58 ± 3.45 g dry mass/L (Fig. 5*a*) was obtained. The pH of the medium fell gradually as the fermentation time increased and maximum CPT was detected at pH 3.5 (Fig. 5*b*). Dissolved oxygen decreased to 56% saturation at the end of fermentation. The residual sugar concentration of 1% was observed at the time of maximum production of CPT (Fig. 5*c*). There was a steady increase in the growth of mycelium from days 1–4 in the bioreactor, but maximum CPT was recorded at 48 h of fermentation (Fig. 5*d*), which indicates that production of CPT started immediately after incubation when sugar percentage also started declining. In shake flasks the maximum CPT was recorded at 96 h of fermentation. The production of the CPT by the culture started declining after day 4 and there was almost a stationary phase of CPT production after day 7 of fermentation in shake flasks. The highest CPT content in the bioreactor culture (4.96 ± 0.73 mg/100 g dry mycelium at 48 h) was several-fold higher than the respective content in the shake-flask culture (0.575 ± 0.031 mg/100 g dry mycelium at 96 h). As it is generally possible to achieve an adequate aeration and mixing in bioreactor cultures, the higher CPT production yields could be the result of an appropriate combination of all the above given factors.

The study indicates that the endophyte *E. infrequens* may be a potential organism for further development and optimization of a fermentation process for the production of CPT and its derivatives as an alternate source to *N. foetida* roots.

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Separation of 9-Methoxycamptothecin and Camptothecin from *Nothapodytes foetida* by Semipreparative HPLC

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Abstract

The present work describes the isolation of camptothecin and 9-methoxycamptothecin from the aerial parts of *Nothapodytes foetida* by semipreparative high-performance liquid chromatography because the separation of compounds by conventional procedures is tedious and cumbersome. The purity of the isolates is determined by physicochemical data and liquid chromatography–mass spectrometry.

Introduction

Nothapodytes foetida Sleumer or *Mappia foetida* Miers (commonly called "Kalgur") belongs to the family *Icacinaceae*. This small tree, whose extract shows promising anticancer activity, is distributed in the western Indian peninsula from Konkan southwards in the areas of the Nilgiris and Konkan Ghats. *N. foetida*, along with other species, is a rich source of camptothecin (CPT) (Figure 1) and minor camptothecinoids (1–3). CPT has regained its position as a leading molecule in cancer chemotherapy with the development of certain derivatives [such as irinotecan, topotecan, exetecan, 9-amino, and 9-nitro-20 (S) CPT], which are under clinical trails and have promising chemotherapeutic efficacy. CPT and its analogues have a unique mechanism of action; they produce DNA damage in the presence of topoisomerase-I by binding to and stabilizing a covalent DNA-topoisomerase-I complex in which one strand of DNA gets broken.

N. foetida plants have been successfully raised from their seeds in the agro-climatic region of the northwestern Himalayas (4). Earlier reported procedures for isolation of 9-methoxycamptothecin from the crude extract involved cumbersome procedures encompassing high-speed counter-current chromatography and repeated crystallizations (1,5). In the present methodology, 9-methoxycamptothecin has been isolated in 95% purity by semipreparative high-performance liquid chromatography (HPLC) from the mother liquor after crystallization of CPT from the crude extract of *N. foetida*.

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Experimental

Chemicals

HPLC-grade acetonitrile and water were obtained from Ranbaxy (Mohali Chandigar, India).

Chromatographic conditions

Analytical HPLC instrumentation

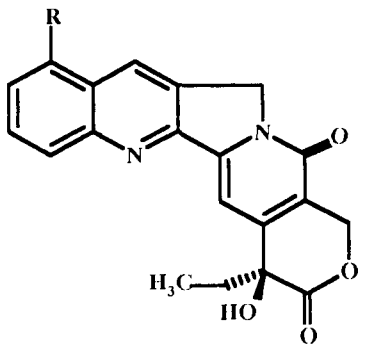
Analytical HPLC was performed on a Gilson (Villiers Le Bel, France) HPLC that included a 305 pump and 10SC pump head, 306 manometric module, 115 UV detector set at 256 nm, 7725i injector with a 50-µL sample loop (Rheodyne, Cotati, CA), and Lichrosphere RP-18 column (4- × 100-mm, 5-µm particle size) (Merck, Darmstadt, Germany) to separate the compounds.

Semipreparative HPLC instrumentation

A Gilson semipreparative HPLC system consisting of 305 pump and 25SC pump head, 306 manometric module, Rheodyne 7725i injector with 300-µL sample loop, 116 UV detector set at 272 nm, and Rainin Dynamax semiprep C-18 column (100- × 5-mm, 2-µm particle size) was used along with Gilson 201 fraction collector.

Liquid chromatography–mass spectrometry

Liquid chromatography (LC)–mass spectrometry (MS) experi-



CPT I R = H
9-Methoxycamptothecin II R = OMe

Figure 1. The structure of CPT.

ments were performed on a Bruker (Bremen, Germany) Esquire 3000 ion trap MS with an electrospray ionization (ESI) interface and connected to an Agilent (1100 series) binary pump, photodiode array detector, automatic sample injection module, and thermostatic column oven (Agilent, Palo Alto, CA).

Isolation of 9-methoxycamptothecin and CPT and processing of collected fractions

Plant material was collected from RRL (J) botanical garden (Jammu, India) and cultivated from the seeds obtained from the Mahabaleshwar forests of India. A finely powdered aerial portion of plant material (100 g) was extracted with methanol in a Soxhlet apparatus. The extract was filtered and concentrated on a rotatory evaporator. The crude extract (9.5 g) was suspended in 100 mL of water, which was further partitioned with petroleum ether (3 × 50 mL) and chloroform (3 × 50 mL), successively. The chloroform extract was dried over anhydrous sodium sulphate, concentrated on a rota-evaporator, and dissolved in boiling chloroform–methanol (80:20 v/v). As a clear solution standing for 4–6 h at 5°C, CPT precipitates out as a fine, yellow powder.

Mother liquor (10 mg) was dissolved in 10 mL of a chloroform–methanol mixture (80:20 v/v) and subjected to an analytical HPLC system with UV detector set at 256 nm (Figure 2). The mobile phase consisting of premixed water–acetonitrile (25:75) [filtered and degassed on Millex HV filter (0.45 µm, Millipore, Billerica, MA)] was injected and eluted at a flow rate of 1 mL/min. The chromatogram of the filtered extract was plotted on an HP 3395 integrator (Palo Alto, CA). The peaks of CPT and 9-methoxycamptothecin were detected at retention times (t_R) of 13.079 and 22.13 min, respectively, and confirmed by cospiking with their corresponding standards.

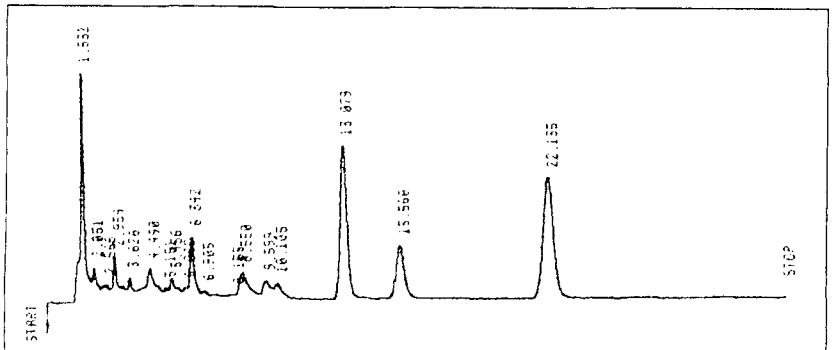


Figure 2. HPLC chromatogram of the crude extract of *N. foetida*. The t_R of CPT is 13.079 min and 9-methoxycamptothecin is 22.135 min.

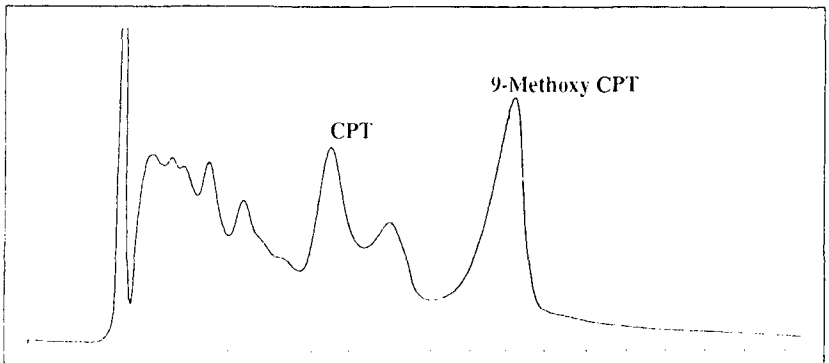


Figure 3. Semipreparative HPLC profile of crude extract of *N. foetida*. Each division on the *x*-axis depicts a time interval of 2 min.

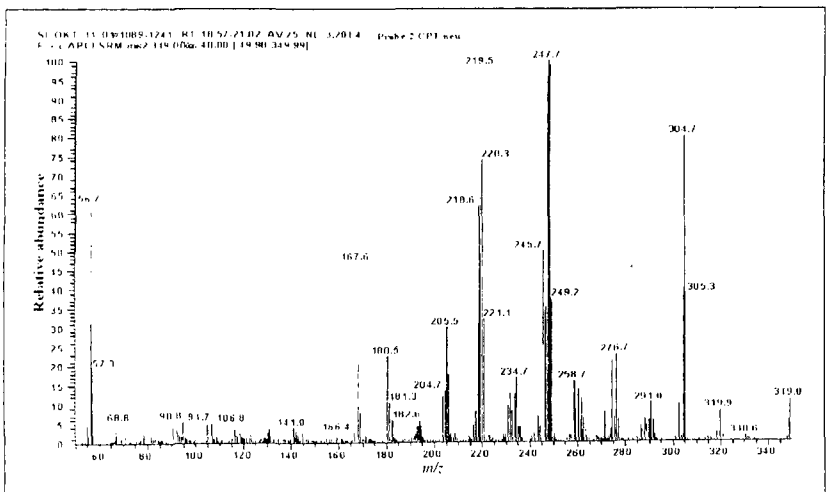


Figure 4. Mass spectrum of CPT.

Semipreparative HPLC of the extract

The mother liquor of the extract (432 mg) was subjected to semipreparative HPLC. A premixed solvent system consisting of water–acetronitrile (25:75) was isocratically pumped at a flow rate of 3 mL/min. Extract (300 µL) was loaded on the Rheodyne injector. Two peaks were collected in clean, preweighed flasks. CPT (I) was collected from 14.5 to 16 min and 9-methoxycamptothecin (II) was collected from 23 to 25 min (Figure 3). After 20 such collections, the pooled eluates in an Erlenmeyer flask were visualized under UV showing blue fluorescence in fraction (I) and yellow in fraction (II). The azeotrope phase was removed by rotatory evaporation under reduced pressure. Removal of the organic phase from the respective pooled fractions yielded CPT (15 mg) and 9-methoxycamptothecin (42 mg), respectively.

Purity check of the collected fractions

To check the purity, the residue was dissolved in chloroform–methanol (80:20 v/v). The prepared solution was analyzed on an HP-100 HPLC system (Agilent) linked to a Bruker Daltonics Esquire 3000 MS with an ESI source (LC–ESI-MS–MS) scanned over a mass range between *m/z* 100 and 900. Fraction (I) and (II) solutions (10 µL) were injected separately with an autoinjector on a Merck Chromolith C_{18} column (100 × 4 mm) and eluted with water–acetonitrile (25:75) at a flow rate of 0.8 mL/min. The fraction (I) eluting at a t_R of 13.08 min exhibited molecular adduct $(M+H)^-$ at *m/z* 349.1 (Figure 4). The fraction (II) eluting at a t_R of 22.14 min exhibited molecular adduct $(M+H)^+$ at *m/z* 379.1.

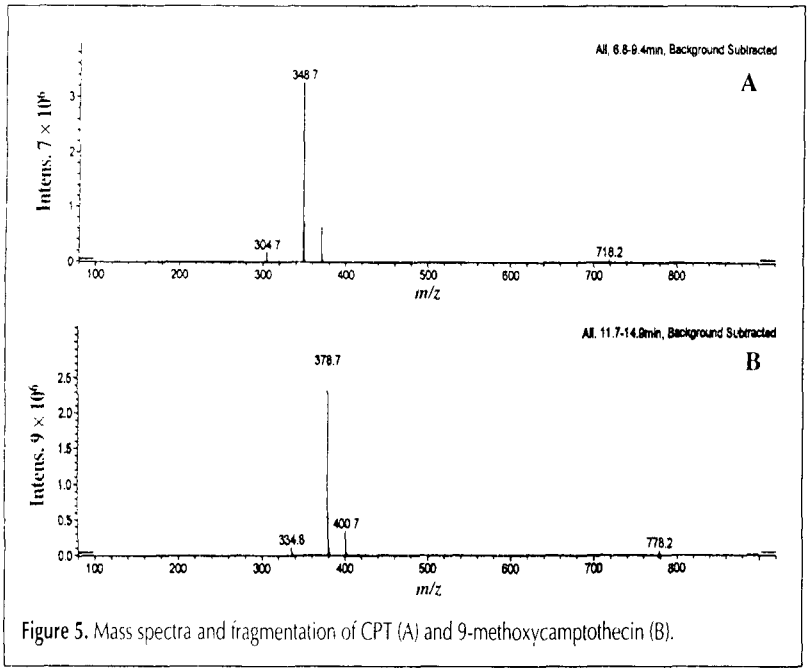


Figure 5. Mass spectra and fragmentation of CPT (A) and 9-methoxycamptothecin (B).

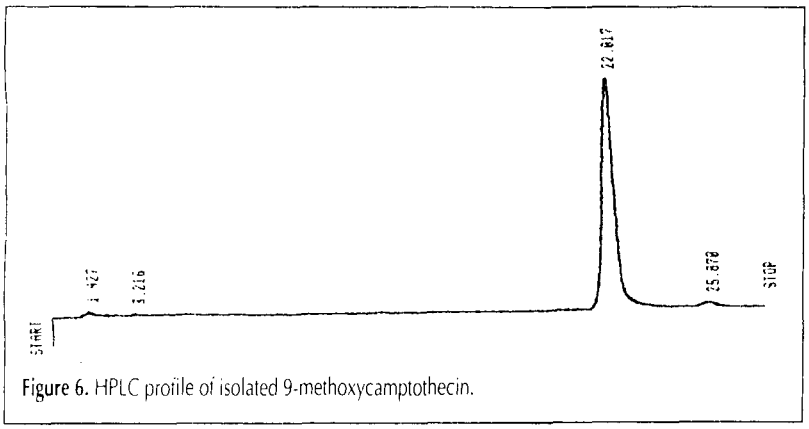


Figure 6. HPLC profile of isolated 9-methoxycamptothecin.

Results and Discussion

The purity of collected fractions was determined separately on the basis of LC runs. CPT and 9-methoxycamptothecin eluted at a t_R of 13.08 and 22.14 min, respectively, were confirmed simultaneously by comparison of a total ion chromatogram (TIC) with a LC–UV chromatogram. Complete overlap of the TIC and UV chromatograms pointed out the purity of the collected compounds.

Fraction (I) CPT (95% pure) was crystallized from chloroform–methanol (80:20 v/v) to give light yellow crystals with a melting point of 273–274°C, $C_{20}H_{16}N_2O_4$; $[M+H]^+$ 349.1 (calculated CHN 348.11), $[a]_D^{25}$ = +34.8 (*c* 0.40, 8:2 $CHCl_3$–MeOH; literature +35°). Analytical and spectral data were in agreement with those reported in the literature (6,7). Fraction (II) on crystallization from chloroform–methanol (80:20 v/v) gave yellow crystals: melting point 266–269°C, $C_{21}H_{18}N_2O_5$; M^+ 378 (calculated CHN 378.38) $[a]_D^{25}$ = –98.54 (concentration of compounds in pyridine 0.29). Thin-layer chromatography, UV, HPLC, and LC–MS data (Figure 5) confirmed its identity and purity (95%) as 9-methoxycamptothecin (Figure 6).

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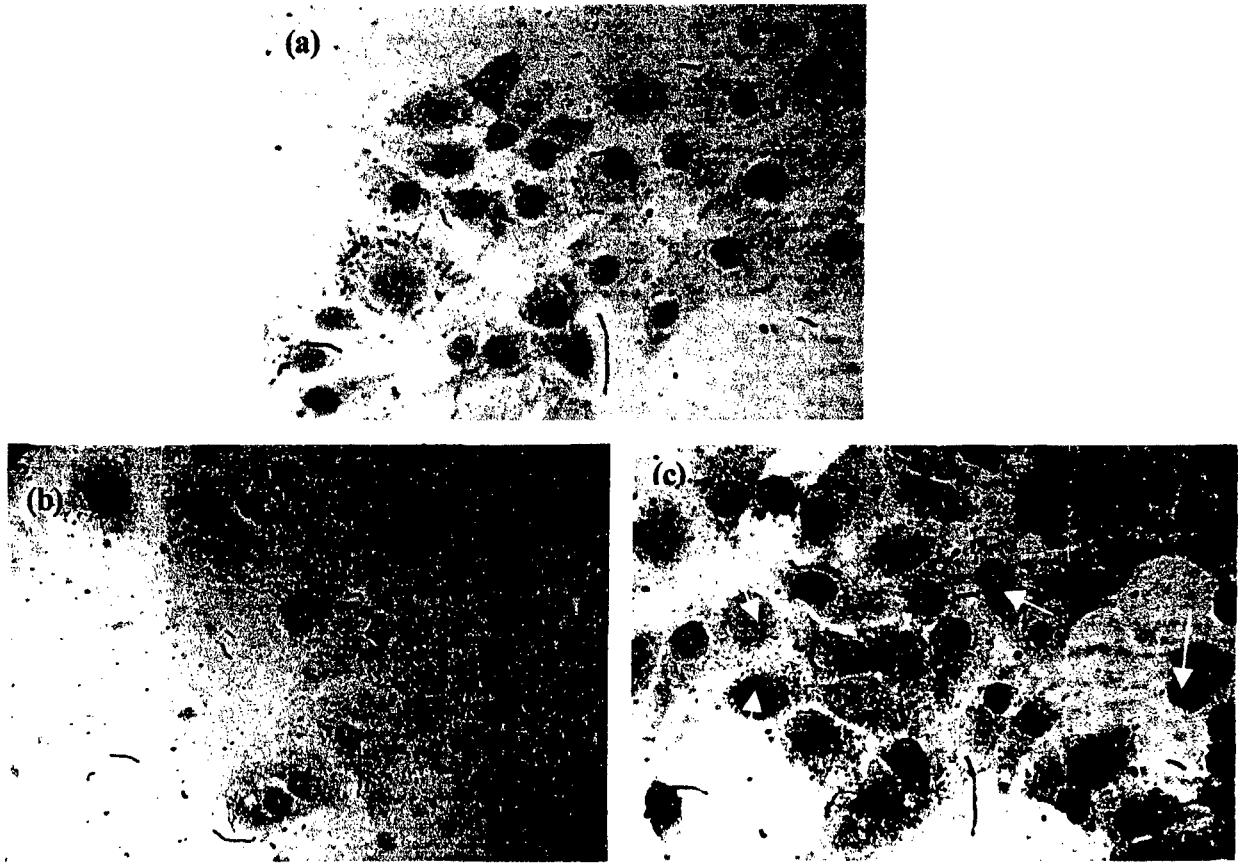

Fig. 43: (a-c) Hep-2 cells. (a) Untreated, (b) Mitomycin-C treated cells. Mag. 450X (c) CPT treated Hep-2 cells ($1x10^{-5}$ M) show retraction of protoplasmic extensions, condensation and marginalization of chromatin material in the nuclei (arrow) Mag. 450X

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The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans

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Abstract

The aryl tetralin lignans are synthesized by *Podophyllum* sps. and are in great demand worldwide due to their use in synthesis of topoisomerase inhibitors. However, the sustained production of these aryl tetralin lignans requires large-scale harvesting from the natural environments, which has resulted in the plant-endangered status. In view of the difficulties in their total chemical synthesis, cultivation and failure of metabolic engineering approaches, there is a need to search for alternative sources of production of aryl tetralin lignans. We unequivocally established the methodology for isolation, identification, and characterization of a novel fungal endophyte (*Trametes hirsuta*) that produces aryl tetralin lignans consistently as shown by HPLC, LC–MS, LC/MS–MS and ^{1}H NMR. The lignans produced by the microorganism are biologically active, and exhibit potent antioxidant, anticancer and radioprotective properties. This strategy promises to improve the production of these therapeutically important compounds at lower costs.

Keywords: *Podophyllum hexandrum*; *Trametes hirusta*; Endophyte; Radioprotection; Aryl tetralin lignans; Podophyllotoxin

1. Introduction

Natural products represent an invaluable gold mine of pharmcophores for pharmaceutical and agricultural industry sector (Wall and Wani, 1996). A plethora of natural products from microbial, plant, and animal sources have been evaluated for their diverse

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biological activities including anticancer, antioxidant, antiviral, immunostimulant and radioprotective properties (Weiss and Landauer, 2003; Arora et al., 2005a,b,c).

The plant *Podophyllum* (Mayapple) synthesizes podophyllotoxin, 4′-demethylpodophyllotoxin, podophyllotoxin glycoside and other polyphenolic compounds that find application as anticancer, antiviral, antibacterial, immunostimulating, and anti-rheumatic drugs (Singh and Shah, 1994; Pugh et al., 2001). The demand for these compounds is steadily increasing world-wide due their increasing use in preparation of semi-synthetic topoisomerase inhibitors (Eich et al., 1991). Our previous studies on *Podophyllum hexandrum* have proved the radioprotective property of aryl tetralin lignans (Gupta et al., 2003; Chawla et al., 2005a,b). However, there are several constraints in meeting the high volume of demand of these compounds, e.g., natural populations of *Podophyllum* are fast depleting due to over-harvesting, and consequently the plant has been declared as an endangered species (Foster, 1993). Agricultural production has been unsuccessful since the plant is not amenable to cultivation in the absence of proper climatic conditions (Moraes et al., 2001; Lee and Xiao, 2003). Metabolic engineering approaches have not been feasible since the enzymes or genes involved in podophyllotoxin (PDT) biosynthesis are not precisely known. Other biotechnological approaches, e.g., cell/tissue culture have also not yielded desirable results (Berlin et al., 1988; Empt et al., 2001; Giri and Narasu, 2000; Peterson and Alferman, 2001). Total chemical synthesis of these bioactive lignans is not commercially feasible due to the presence of four chiral centers along with a γ-lactone and a high degree of oxygenation (Damayanti and Lown, 1998; Berkovitz et al., 2000).

Attempts for development of alternative sources for the production of aryl tetralin lignans has therefore, resulted (Bhadula et al., 1996) in little success. For establishing an inexhaustible, cost effective and a renewable resource of the low-volume, high-value aryl tetralin lignans, fermentation technology (involving a microbe) appears promising since industrial production requires reproducible and dependable productivity. In the search for an alternative viable source of bioactive compounds of *Podophyllum*, we evaluated the high-altitude *P. hexandrum* that grows in virgin Himalayan environments that provide an ideal ground for the formation of host–microbe relationships.

In this study, we demonstrate the isolation of an endophytic fungus *Trametes hirsuta*, from *P. hexandrum* that specifically produces podophyllotoxin. Growth and production under fermentation conditions was standardized and chemical characterization performed using modern chromatographic and spectroscopic methods. The novel endophyte can be utilized for its fermentation and biotechnology capabilities as an alternative mode of production of the bioactive moieties. The isolated endophyte can be easily grown in vitro, biomass accumulation is rapid, is amenable to up-scaling, and augmentation of secondary metabolites is possible, which is independent of the vagaries of environment (unlike in plants). Furthermore, the podophyllotoxin and its glycoside isolated from this source has been found to be biologically active, i.e., possesses antioxidant and radioprotective properties. Spectral data, obtained using HPLC, LC–MS, MS/MS and ^{1}H NMR, of the fungal secondary metabolites, including podophyllotoxin, and its glycoside is identical to authentic molecule(s).

2. Materials and methods

2.1. Collection, identification and authentication of the plant material

P. hexandrum was collected from the north-western Himalayan region of Jammu and Kashmir, India in the month of April. Voucher specimen has been deposited in the repository at RRL, Jammu (Voucher Specimen No. RRL(J)-Endo-SCP-ANZR/RA). The plant material was identified and authenticated on the basis of botanical characteristics by an experienced botanist.

2.2. Isolation and establishment of in vitro culture of the endophyte

The endophyte was isolated from the dried rhizomes of *P. hexandrum*, using a modified method described by Schulz et al. (Arnold et al., 2000). Pieces of rhizome were thoroughly washed using distilled water and followed by 70% (v/v) ethanol for 1 min and 5% sodium hypochlorite for 5 min to accomplish surface sterilization. They were subsequently rinsed in sterile deminer-

alized water thrice for 1 min. Small pieces (1 cm) of inner tissue of rhizome were placed on aqueous agar in petriplates (Tarsons, Kolkata, India) and incubated at 28 ± 20 °C until the fungal growth was initiated. The tips of the fungal hyphae were then removed from the aqueous agar and placed on a mycological medium. The pure endophytic fungal culture, thus obtained, was transferred on to a number of solid and liquid media, which supported the fungal growth. Similar procedure, but without surface sterilization, was used as a negative control to check for surface contaminated fungi. The detailed procedure for endophyte isolation was verified using the vitality test (Empt et al., 2001). To establish the de novo production of podophyllotoxin by the isolated fungi, the growing mycelium was serially transferred several times to fresh mycological agar to eliminate the possibility of the fungal hyphae carrying even minute amounts of podophyllotoxin and their glycosides as 'contaminant' from the initial plant material.

2.3. Identification and authentication of endophyte using homology modeling

Microscopic slides were prepared, stained using lactophenol cotton blue (Vainio et al., 1998) and were examined under light microscope (Olympus, USA). Photographs were taken by using a digital camera (Camedia camera, C-2100 ultra zoom, Tokyo, Japan). The total genomic fungal DNA was extracted by CTAB method (Cappiccino and Sherman, 1996). For DNA extraction, the fungus was grown in 100 ml Sabouraud Dextrose Agar at 28 °C with constant shaking for 3 days. One hundred milligrams mycelial biomass was taken following washing (two times) with sterile Tris–EDTA buffer, 6 ml of CTAB extraction buffer and 60 μl of β-mercaptoethanol were added. After mixing the mixture was incubated at 65 °C for 45 min, and the contents cooled to room temperature. This was followed by extraction with equal volume of chloroform and centrifugation at 10,000 × *g* for 10 min. Equal volume of isopropanol was added to the supernatant and mixed gently. The DNA pellet was washed with ice-cold 70% (v/v) ethanol. The DNA pellet was vacuum dried and dissolved in 100 μl of TE (pH 8.0). The endophytic fungus was identified by analysis of the large and small subunit of ribosomal genes. The large subunit of ribosomal gene was amplified and sequenced using MICROSEQ D2, large subunit (LSU ~ 300 bp) fungal rDNA sequencing kit (Applied Biosystems, USA). The small subunit ribosomal gene was amplified with primers 5′ TCCGTAGGTGAACCTGCGG 3′ and 5′ TCCTCCGCTTATTGATATGC 3′. The amplified products were purified utilizing Microcon columns (Millipore, USA), and sequenced using ABI Prism310 genetic analyzer (ABI, USA) as per the manufacturer's instructions. The DNA sequences ~300 and 500 bases, thus obtained were submitted to Genbank for homology studies by BLASTN program (Altschul et al., 1997). The ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) was accessed and sequence alignment was used as an underlying basis to identify the fungus (Gene accession nos. AY972129 ITS 1, 5.8S; AY972128 25S). A sample of the fungus (Fig. 1) has been deposited in the Repository at the Institute of Microbial Technology, Chandigarh, India (Microbial Type Culture Collection (MTCC), accession no. yet to be assigned).

2.4. Maintenance of growth of the endophytic organism

The endophyte was grown in Sabouraud broth consisting of dextrose (4%), and peptone (1%). Agar blocks, impregnated with mycelia, were used as inoculums. The endophyte was grown in 500 ml Erlenmeyer flasks each containing 100 ml liquid broth (pH 5.6) for a period of 7 days at 28 ± 2 °C, on an incubatory shaker (New Brunswick, USA; 220 rpm). The first sample was collected immediately after inoculation (0 h) and subsequent samples were collected every 24 h up to 7 days. The samples were filtered to separate mycelia and broth, and the fresh biomass was determined. After repeated washing with sterile demineralized water to remove the residual medium. The moisture content, final pH and dry weight was also determined for each sample. The experiments were performed in triplicate and were repeated six times.

2.5. Isolation, identification and authentication of aryl tetralin lignans

The mycelia were thoroughly washed with sterile demineralised water and sonicated (MSE Hanor Roya Crawley RH10 2QQ cell disrupter). Cell homogenates were then extracted four times with a mixture of

chloroform:methanol (4:1 v/v). Solvent was stripped off in a rotary evaporator leaving behind the organic residue, which, was dissolved in methanol and placed on a chromatographic glass column of neutral alumina. Elution was carried out using different solvents in gradient, viz., *n*-hexane, chloroform, mixture of chloroform:methanol and methanol successively. A compound having same chromatographic mobility

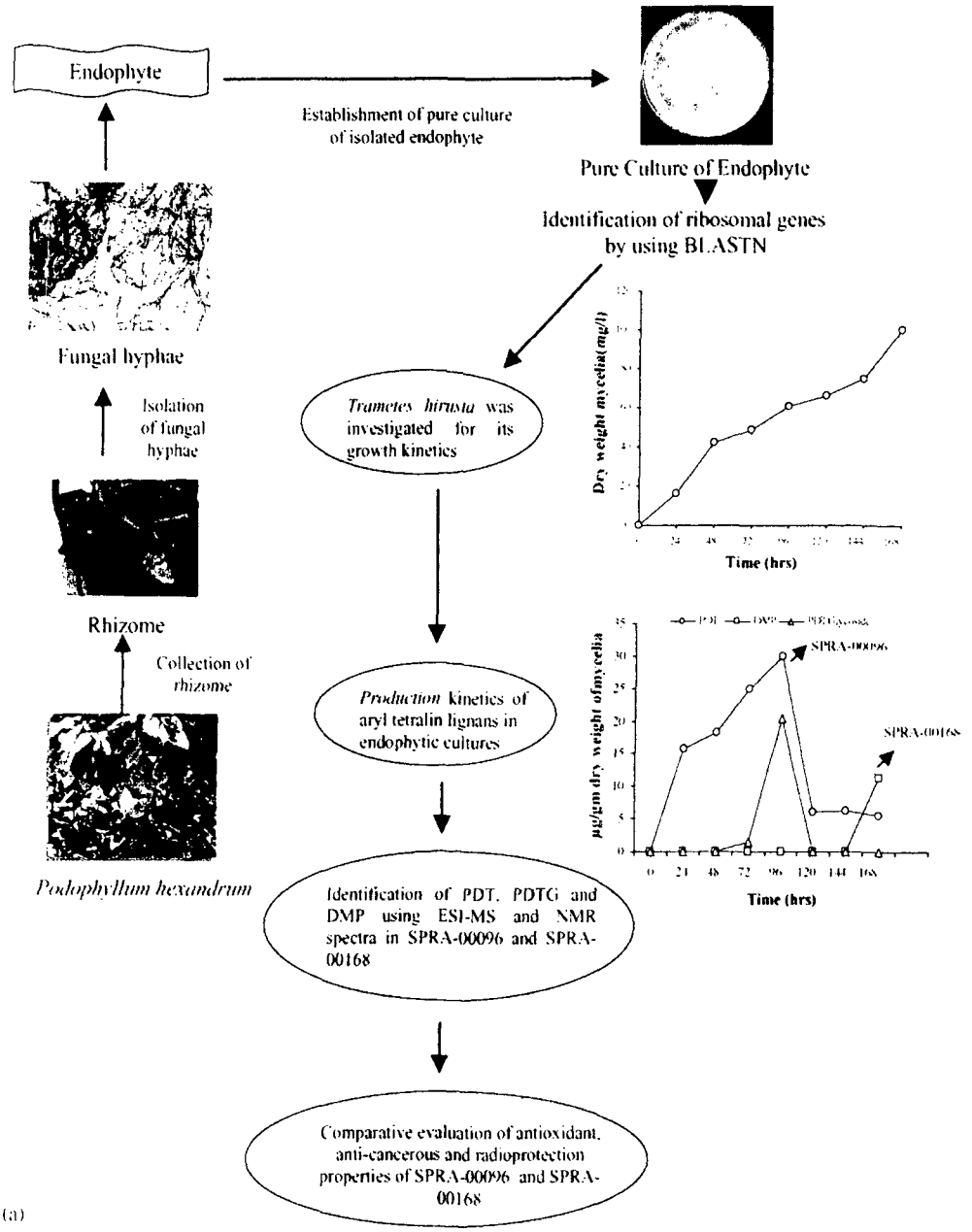
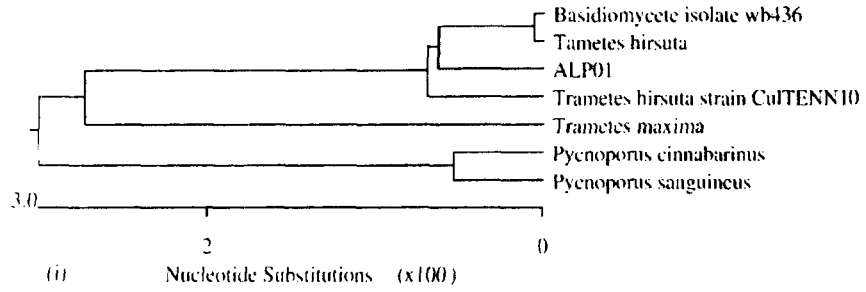


Fig. 1. (a) Schematic representation of the experimental strategy adopted for isolation, identification and maintenance of culture of an endophyte associated with *P. hexandrum* (>3000 m above the sea level). The fractions collected at 96 and 168 h was compared for their biological activities and compared with their relative content of PDT, PDTG and DMP. (b)—(i) Dendrogram showing the phylogenetic position of isolate, generated by alignment of ribosomal gene sequences using Clustal W and (ii) pairwise distance matrix of ribosomal gene sequences of different isolates showing the phylogenetic position of strain ALP01.



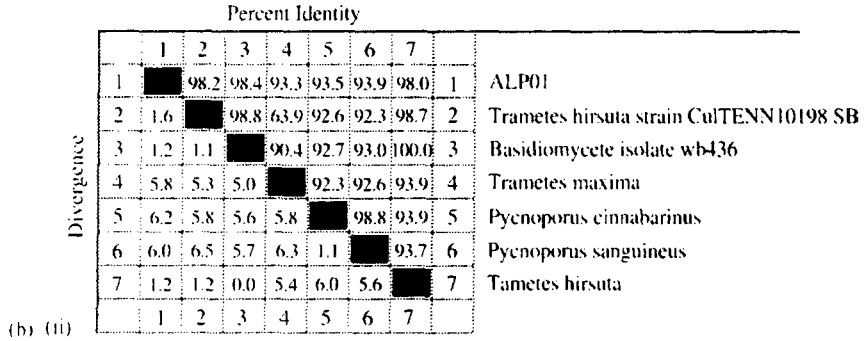


Fig. 1. (*Continued*).

(R_f = 3.5) as authentic PDT was found in the chloroform:methanol (4:1) fraction. The identity of different constituents was confirmed by TLC, HPLC, LC–MS, LC/MS–MS and ^{1}H NMR (Table 1).

2.5.1. Thin layer chromatographic analysis

All comparative TLC analyses were carried out on Merck 0.25 mm silica gel plates 1 mm (20 cm × 20 cm) developed in a solvent system comprising of chloro-

Table 1
Physico-chemical characteristics of the fungal lignan and its glycosides

Analytical data	Fungal podophyllotoxin	Fungal-podophyllotoxin glycoside
m.p. (°C)	182–184	172–176
TLC (R_f) ($CHCl_3$:MeOH 25:1)	0.2	0.35
HPLC (R_t) (min) (mobile phase isocratic system methanol:H_2O, 65:35), column RP-18 and 5 μm (E. Merck) flow rate: 0.6 /min, λ_{max}: 290	8.78	6.57 and 7.71
LC/MS (R_t/MS) (mobile phase MeOH:H_2O 65:35), flow rate 0.6 ml/min λ_{max}: 290	R_t 6.2, 7.1 7.7, 23.7 and 26.7 min, *m/z* 437.2 (M + Na) (R_t 7.7 min), *m/z* 437 (M + Na), 414, 397 ($M - H_2O$), 313 ($M - 84$), 247 ($M - 168$) and 229.1 ($M - 168 - 18$)	R_t 4.7 and 5.5 min, *m/z* 599.2 (epi-isomer), 599.2 (M + Na), 437.03, 412.03, 396.99, 352.96, 312.94 and 245.85
LC/MS–MS	*m/z* 437 (M + Na), *m/z* 419 ((M + Na) − 18)	*m/z* 599.15 (M + Na), 414.15, 352.96, 312.94
IR (KBr) ν_{max}^{KBr}	3610, 3515, 1770, 1590, 1508	3370, 1768, 1588, 1503, 1490
Optical rotation	$[\alpha]_D^{20} = -190°$ (C.0.073 $CHCl_3$)	$[\alpha]_D^{23} = -89.2°$ (C.0.57 MeOH)
^{1}H NMR $CDCl_3$, 500 MHz	Alicyclic protons; δ 4.75 (1H, d, J = 8.8 H-4), 4.58 (1H, m), 4.59 (1H, m, H-3aα), 4.07 (1H, d, J = 9.5, H-3aβ), 2.9 (1H, s, H-2) and 2.7 (1H, m, H-3), aromatic protons 7.12 (H-5), 6.51 (H-8), 6.37 (H-2′ and H-6′), 5.99 and 5.97 (d, J = 1.2), 3.81 (3H 4′-OMe and 3.75 (6H, 3′,5′-OMe)	Agylcones protons along with glycosides protons; glucose C-1″-104.69, C-2″-75.20, C-3″-78.00, C-4″-71.22, C-5″-77.06, C-6″-68.56

form:methanol (25:1). The plate was visualized under UV (254, 365 nm) radiation. Standard podophyllotoxin was also run along with the samples. Podophyllotoxin and PDT glycoside exhibited R_f values of 0.2 and 0.35, respectively. TLC spots with R_f values identical to that of podophyllotoxin and its glycoside were eluted with methanol for HPLC and LC–MS.

2.5.2. *High performance liquid chromatographic analysis*

HPLC separation was performed isocratically using Luna RP-18 column (3 μm, 150 mm × 2 mm) and employing a safety guard (Phenomenex, Torrance, CA, USA) at 30 °C. The mobile phase was set as methanol:water, 65:35 and separation was carried out at a flow rate of 0.6 ml/min over a period of 60 min and then set at 35:65 up to 70 min. The compounds were detected using a UV detector at λ_{max} of 290 nm.

2.5.3. *APCI-LC/MS/MS*

The operating conditions were optimized for the MS–MS analysis of arylnapthalene and related lignans. LC–MS was carried out on a Bruker–Bremer system, Germany. All experiments were performed using a Thermo-Finagan MAT8000 pneumatically assisted electrospray triple–quadruple mass spectrometer. The nebulizer curtain and collision gases were set at 1.25, 0.45 and 0.4 l/min, respectively, and the gas was supplied from a liquid nitrogen tank with a head pressure of 20 psi. The ion spray voltage was 4800 V. The voltage at the orifice plates focusing ring deflector and channel electron multiplier (CEM) was adjusted at 350–400 and 2100 V, respectively. Positive ions were scanned in the range 350–700 dalton (Da) using 10 ms dwell time and a 0.2 Da step size during scans.

2.5.4. *Optical rotation*

Instrument. Perkin-Elmer Polarimeter. Tuned with 10 cm cell path length using solvent chloroform:methanol (8:2).

2.5.5. *ESI ionisation and MS/MS*

Instrument. Thermo Finnagan TSQ7000. The optical collision energy was determined by means of an ICL procedure controlling the automatic switching between different voltages with a step size of 0.5 V/scan to 40 V. During this procedure, the analytes were injected via a Rheodyne valve with a 2 μl injection loop at a concentration of 10 μg/ml. A pre-scan voltage settling time of 20 min and 0.4 s for one complete cycle (and transitions) was used for selected monitoring (SRM). The HPLC and LC/MS and MS/MS fragmentation patterns were identical to the authentic molecules. Peaks showing same molecular weight with that of authentic compounds were isolated through semi-preparative HPLC system and were confirmed with ^{1}H NMR.

2.5.6. *^{1}H nuclear magnetic resonance spectral analysis*

NMR spectra were recorded on a Brucker DPXx200 spectrometer; chemical shifts are given in δ units relative to the tetramethylsilane (TMS) signal as an internal reference coupling constant (J) is reported in Hz.

2.6. *Establishment of secondary metabolite production as a function of time*

The endophyte was grown in Erlenmeyer flasks (500 ml), each containing 100 ml Sabouraud broth, sonicated and extracted by the same procedure as described above. Podophyllotoxin and their corresponding glycosides were quantified by HPLC. Validation of the quantitative method was performed for samples in triplicate. The experiment was repeated six times and data presented as the mean value for each time period. The samples obtained at 96 and 168 h were coded as SPRA-00096 and SPRA-00168, respectively, and further bioactivity evaluation was performed using these samples.

2.7. *Evaluation of hydroxyl ion quenching potential of selected fractions*

Hydroxyl ion scavenging potential of SPRA-00096 and SPRA-00168 was measured using the deoxyribose degradation assay (Stierle et al., 1993). Varying concentrations of SPRA-00096 and SPRA-00168 (500 μl) were mixed with 100 μM each of ferric chloride solution, EDTA, and L-Ascorbic acid along with H_2O_2 (1 mM), deoxyribose (3.6 mM) in potassium phosphate buffer (pH 7.4). The total assay volume was adjusted to 1 ml and the assay mixture was incubated for 1 h at room temperature. In site-specific assay, in lieu of EDTA, equal volume of buffer was used. One milliliter each of TCA (10%, w/v) and thiobarbituric acid (0.5% (w/v), TBA in 0.025 M NaOH) were added to each

sample and incubated in a hot water bath (55 °C) for 15 min, cooled and the absorbance recorded at 532 nm. The decrease in absorbance at a particular concentration indicated higher hydroxyl ion scavenging potential with respect to control.

2.8. Cytotoxicity analysis

Human malignant glioma cells (U87) were grown in T-25 culture flasks (Nunc) in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) supplemented with 1000 units/ml penicillin and 100 μg/ml streptomycin sulfate, in a humidified atmosphere in 5% CO_2 at 37 °C. For experimentation, the exponentially growing cells (1×10^3) were used. Cell viability of U-87 cell line was monitored using propidium iodide (PI) exclusion and Hoechst-33342 (H-33342) uptake. After various treatments with SPRA-00096 and SPRA-00168, cells were harvested at pre-determined time periods, washed in PBS and centrifuged at 1000 rpm for 10 min to obtain a pellet. Solutions of PI and H-33342 in PBS (100 μg/ml) were prepared separately and were mixed in equal volumes. One milliliter of this solution was used to assess cell viability under fluorescence microscope (Leica, Germany). H-33342 stained cells were considered viable, while cells retaining PI were dead. The surviving fraction revealed the dose-dependent toxicity.

2.9. Evaluation of anti-lipid peroxidation activity against supra-lethal irradiation

2.9.1. Animals

Adult swiss albino Strain 'A' male mice (25 ± 2 g), maintained in the animal house of INMAS, Delhi (India), were utilized for the study. The animals were maintained under controlled temperature (25 ± 2 °C) and were subjected to 12 h alternating dark and light cycle in polypropylene cages. Food and drinking water was provided ad libitum. All experiments were carried out after seeking approval of the Institutional Animals Ethics Committee (IAEC).

2.9.2. Irradiation

Gamma radiation (0.25 kGy) was delivered to mice liver homogenate from a ^{60}Co gamma chamber (Gamma Cell 5000, Bhabha Radiation Isotope Technology, Bombay) at a dose rate of 3.38 kGy/h. Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's chemical Dosimetry method.

2.9.3. Estimation of radiation-induced TBARS

Thiobarbituric acid reactive substances (TBARS) were estimated as per the method of Srour et al. (2000).

3. Results

3.1. Isolation and standardization of the culture of an endophyte from rhizomes of plant

P. hexandrum (Himalayan Mayapple; family: Berberidiaceae) was chosen as a source plant for isolating the endophyte, since this plant grows in unexplored environments (>3000 m) in the high altitude ranges of the Himalayas where there is a possibility of mutualistic interactions between different groups of organisms. Earlier studies had indicated that virgin environments favor such interactions (Arnold et al., 2000). The rhizome of *P. hexandrum* has been reported to be the most vital natural source of the aryl tetralin lignans. The rhizomes of *P. hexandrum* were cut into small pieces, surface sterilized, rinsed and inner tissues were isolated and placed on aqueous agar in petriplates. At 28 ± 2 °C, growth was found to be initiated. The tips of fungal hyphae were placed on mycological medium to obtain pure fungal culture, which was then transferred to a number of solid and liquid media, which supported the fungal growth. We found the selective media supporting the pure culture of fungi and the isolation of endophyte was verified by performing the vitality test. To establish the de novo production of podophyllotoxin by the isolated fungi, the growing mycelium was serially transferred several times to fresh mycological agar to eliminate the possibility of the fungal hyphae even minute amounts of podophyllotoxin and their glycosides as 'contaminant' from the initial plant material.

3.2. Morphological characteristics of the fungus and identification

The endophytic fungus, isolated from the dried rhizomes of *P. hexandrum*, was investigated (Fig. 1a) on the basis of morphological characteristics and fungus ribotyping. The endophyte typically possesses small hyphae, which average 2–3 μm in diameter and grows

as a white colony mycelium, when young (Fig. 1a). The mycelia are thread-like, branched and aseptate. Well-developed mycelium is slow-growing, and spreads on the solid medium within 8–10 days. Even after a long period of incubation, sporulation couldn't be observed in the endophytic culture. After 3 days of growth in Sabouraud Dextrose Agar (with constant shaking) at 28 °C, mycelial biomass could be collected in milligram quantities. This collected biomass fraction was used further for DNA isolation, and the pellet isolated using CTAB method was vacuum dried and dissolved in TE buffer. Analysis of large and small subunit of ribosomal genes was performed using MICROSEQ D2 (fungal rDNA sequencing Kit) and by using primers 5′ TCCGTAGGTGAACCTGCGG 3′ and 5′ TCCTCCGCTTATTGATATGC 3′. The amplified products were purified, sequenced and the DNA sequences (~300 and 500 bases) were submitted to GenBank for homology studies by BLASTN program (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov). Homology modeling, performed using BLASTN program, revealed that the fungal DNA sequences possess 98% homology with the corresponding gene sequences of *T. hirsuta*. The taxonomical position has been illustrated in Fig. 1b.

3.3. Growth kinetics and isolation, identification and quantification of secondary metabolite(s)

The growth kinetics of the endophyte (under the standardized culture conditions described above), which exhibited an exponential increase in dry weight of the mycelia (Fig. 1), up to seventh day of incubation was examined. In order to study the production kinetics of aryl tetralin lignans, the mycelia were collected every 24 h and aryl tetralin lignans were isolated. The washed cell homogenates of mycelia were sonicated and the cell lysate, thus obtained, was extracted using different solvent systems. Out of the different solvent systems tested, chloroform:methanol (4:1 v/v) exhibited production of an organic residue in rotary evaporator with a compound having same chromatographic mobility (R_f = 3.5) as of authentic PDT. This fraction was used for isolation, identification and quantification of numerous aryl tetralin lignans and validation was done by TLC, HPLC, LC–MS, LC/MS–MS and ^{1}H NMR. In order to identify the PDT, its glycoside and demethoxypodophyllotoxin, thin layer chromatographic based analysis was performed, and the spots identified (R_f = 0.2 and 0.35) were collected and extracted with methanol. A modified gradient system of HPLC analysis followed by LC–MS and MS–MS fragmentation patterns resulted in authentication of aryl tetralin lignans. HPLC and LC/MS analyses of fractions collected at 96 and 168 h showed retention times of 8.6, 7.4 and 5.4 min (Fig. 2) that corresponded to that of standard podophyllotoxin, demethoxypodophyllotoxin and podophyllotoxin glycoside (Fig. 2), which was further, confirmed by co-spiking. Further convincing evidence for the identity of podophyllotoxin and its glycoside were obtained by electrospray mass spectrometry. Characteristically, authentic podophyllotoxin, demethoxypodophyllotoxin and glycoside yielded an m/z (M + Na) peak at 437 (M + Na), 423 (M + Na) and 599 (M + Na), respectively. On the other hand, fungal podophyllotoxin, demethoxypodophyllotoxin and podophyllotoxin glycoside (M + Na) also exhibited peaks at 437 (Fig. 3a), 423 (M + Na) (Fig. 3b) and 599 (M + Na) (Fig. 3c), respectively, thereby confirming the production of a diverse array of aryl tetralin lignans by the endophyte. The compound podophyllotoxin and its glycosides, isolated by semi-preparative HPLC were also confirmed with TLC, HPLC, LC–MS and ^{1}H NMR (Table 1). NMR spectrum of the fungal PDT was found to be identical to that of authentic plant-derived podophyllotoxin (Fig. 4). The podophyllotoxin, podophyllotoxin glycoside and demethoxypodophyllotoxin content of the organic extracts of mycelia, collected at period of regular time intervals, were determined to have an insight into the production kinetics over time (Fig. 1). Maximum production of podophyllotoxin was observed on day 4 (96 h) in terms of μg PDT/g dry weight of mycelia, although, its formation started as early as 24 h (Fig. 1). The podophyllotoxin content rapidly declined after 96 h of incubation, while the podophyllotoxin glycoside production, initiated at 72 h of incubation, peaked at 96 h of incubation period. On the other hand, no demethoxypodophyllotoxin production was observed in the initial time. Its formation was observed only in the later stages (168 h of incubation). The mean maximum yield of podophyllotoxin, podophyllotoxin glycoside in SPRA-00096 (fraction collected after 96 h of incubation was designated as SPRA-00096 while fraction collected after 168 h of incubation was designated as

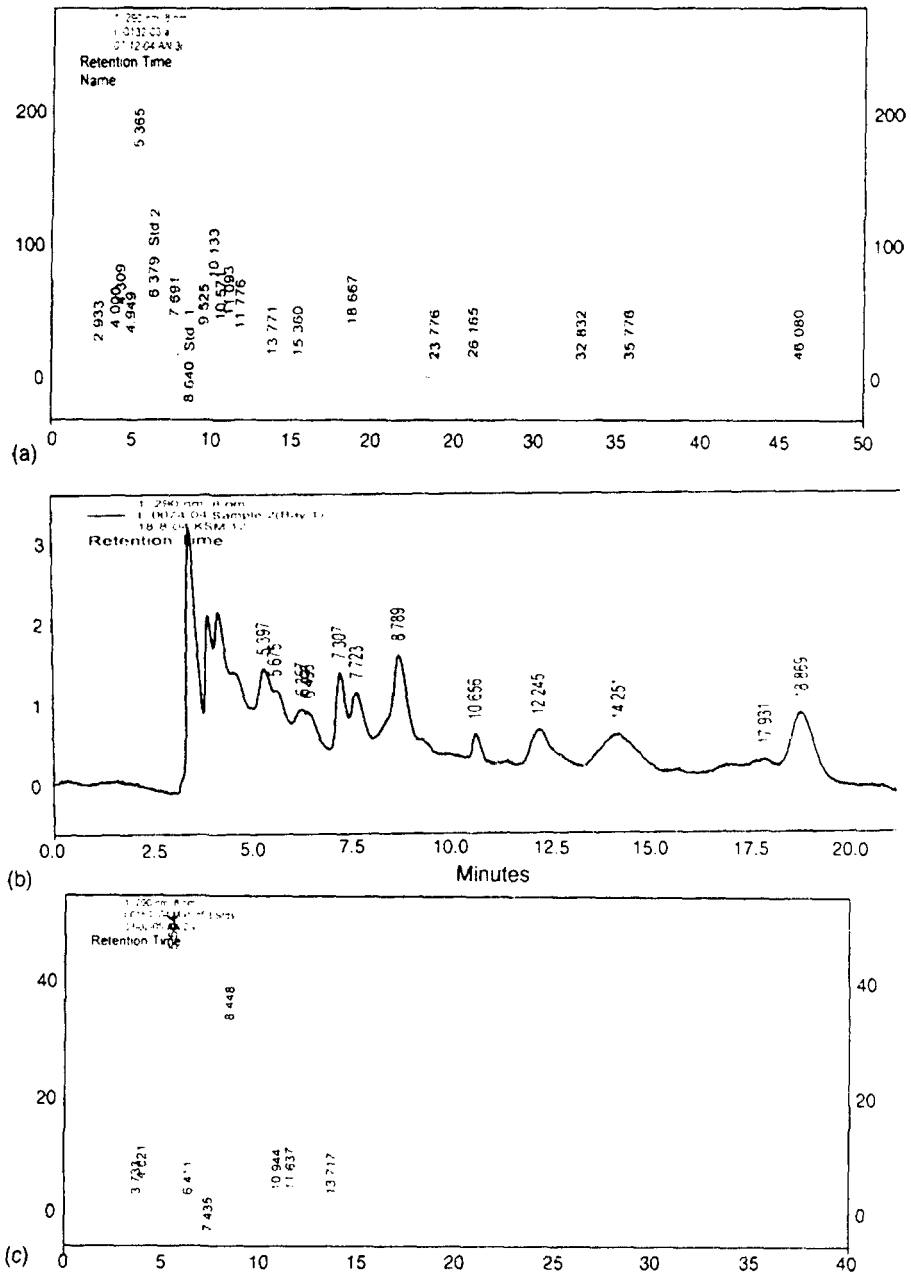


Fig. 2. Identification of aryl tetralin lignans using HPLC: (a) HPLC of SPRA-00096 and (b) HPLC of SPRA-00168. (c) HPLC of authentic compounds, i.e., podophyllotoxin glycoside [PDTG; rt 5.4], podophyllotoxin [PDT; rt 8.4], demethoxypodophyllotoxin [DMP; rt 7.4].

SPRA-00168) was 30 ± 10 μg/g dry weight mycelia, 20 ± 4 and 11 ± 4 μg, respectively. The podophyllotoxin, and podophyllotoxin glycoside were found to be maximum at 96 h of incubation period proving production of both precedes growth. The results of the present study revealed the consistent production of aryl tetralin lignans in cultures. The mean maximum yield of podophyllotoxin, and demethoxypodophyllotoxin in fraction collected at 168 h was 5.5 ± 0.5 μg/g dry weight mycelia, 11 ± 4 μg, respectively. No podophyllotoxin, podophyllotoxin glycoside and demethoxypodophyllotoxin were observed in

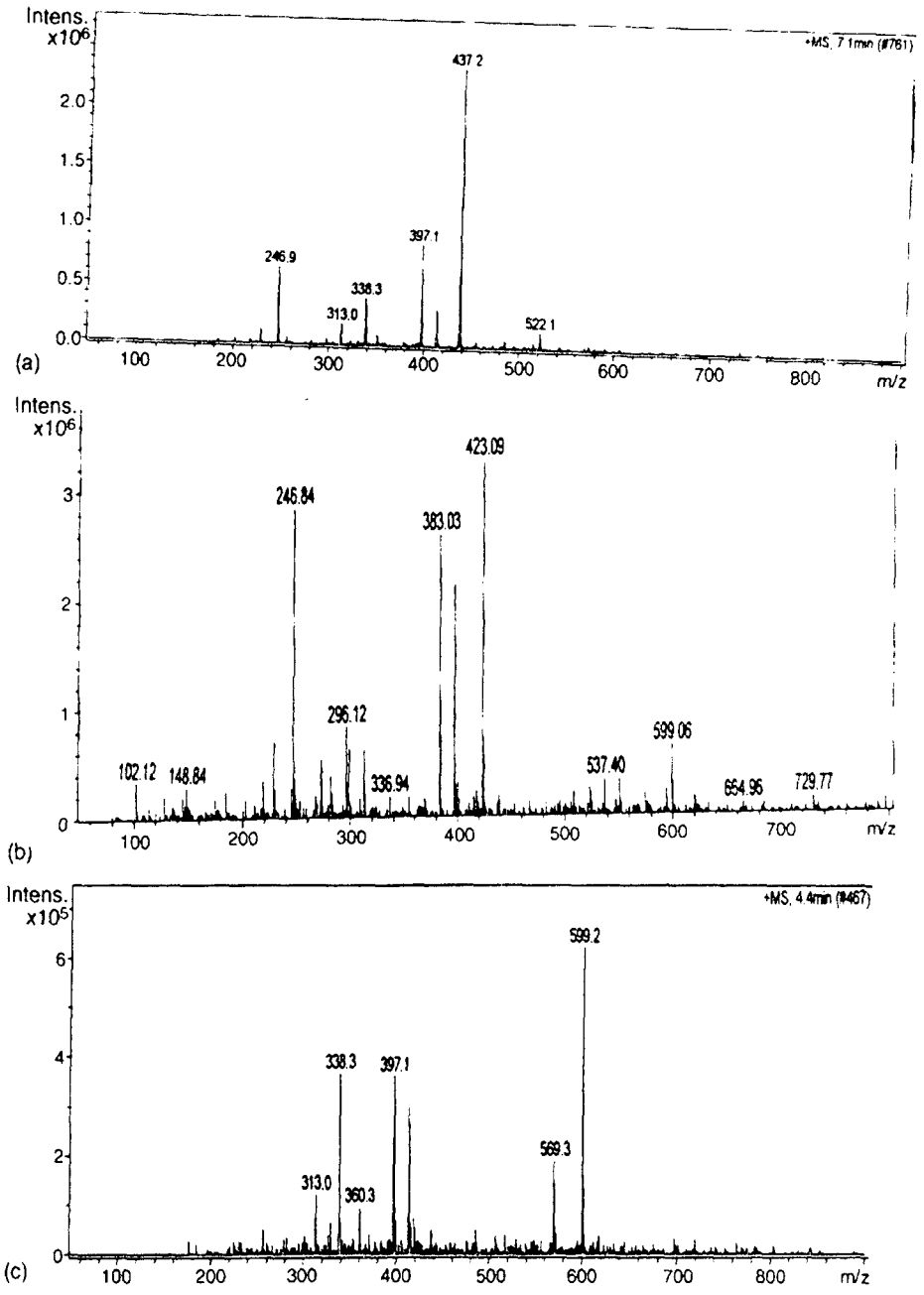


Fig. 3. ESI-MS spectrum of fungal podophyllotoxin (a) demethoxypodophyllotoxin (b) and glycoside (c).

uninoculated culture broths that were extracted with a $CHCl_3$:CH_3OH (4:1 v/v) solvent mixture eliminating the possibility of any cross contamination due to any chance error. The formation of these metabolites was also not observed in inoculated, extracted and processed culture broths at the start of the experiment (0 h). This eliminated the possibility that any lignan had been carried from the original plant material source of the fungus via the mycelium (inoculum plugs). This study thereby established the novel production of the aryl tetralin lignans by the endophyte. The maximum production of aryl tetralin lignans was

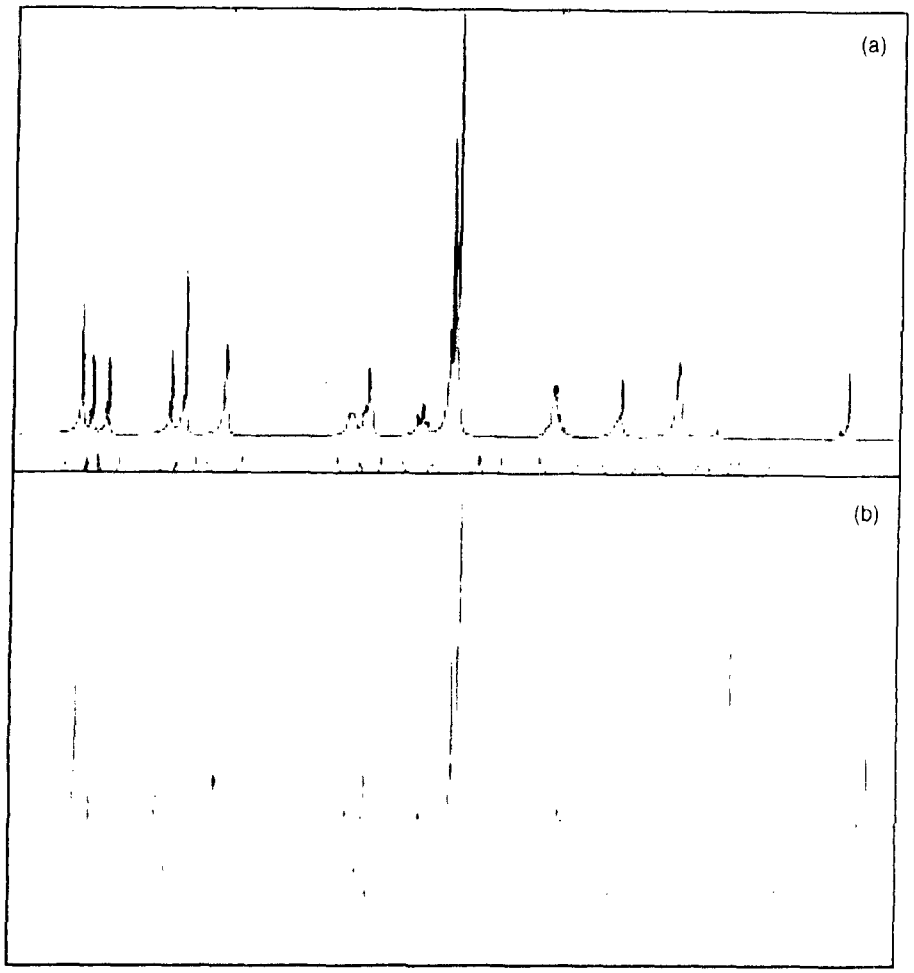


Fig. 4. NMR spectra of (a) authentic podophyllotoxin and (b) fungal podophyllotoxin, *signal merged with $CHCl_3$ impurity in $CDCl_3$ and #hydrocarbon impurity.

observed at 96 h while demethylpodophyllotoxin production was observed after168 h, thus SPRA-00096 and SPRA-00168 were used further for the evaluation of biological activity.

3.4. Selected fractions containing aryl tetralin lignans possess hydroxyl-ion quenching potential

The hydroxyl ion scavenging activity of SPRA-00096 and SPRA-00168, was evaluated using deoxy-D-ribose assay. It was used to study the non- site specific [$Fe^{2+} + H_2O_2$ + EDTA) as well as site-specific [$Fe^{2+} + H_2O_2$] hydroxyl ion scavenging activity in aqueous system. In non-site specific assay, the presence of EDTA makes Fe^{2+} non-available for attacking deoxyribose directly and therefore, hydroxyl generation predominates (Halliwell et al., 1987; Kitts et al., 2000). The hydroxyl ion scavenging potential (drug dose range: 0–100 μg/ml) was evaluated as percentage inhibition of deoxyribose degradation estimated using TBA. The maximum percent inhibition of SPRA-00096 and SPRA-00168 was 32.97% and 20.74%, respectively (Fig. 5). SPRA-00096 exhibited nearly 12% higher non-site specific activity as compared to SPRA-00168, showing its higher ability to directly scavenge hydroxyl ions. The findings of these bioactivity data corroborated well with our earlier reports on the different fractions of the plant (*P. hexandrum*) (Chawla et al., 2005a,b). In a site-specific assay, Fe^{2+} induces hydroxyl generation but maximally attack

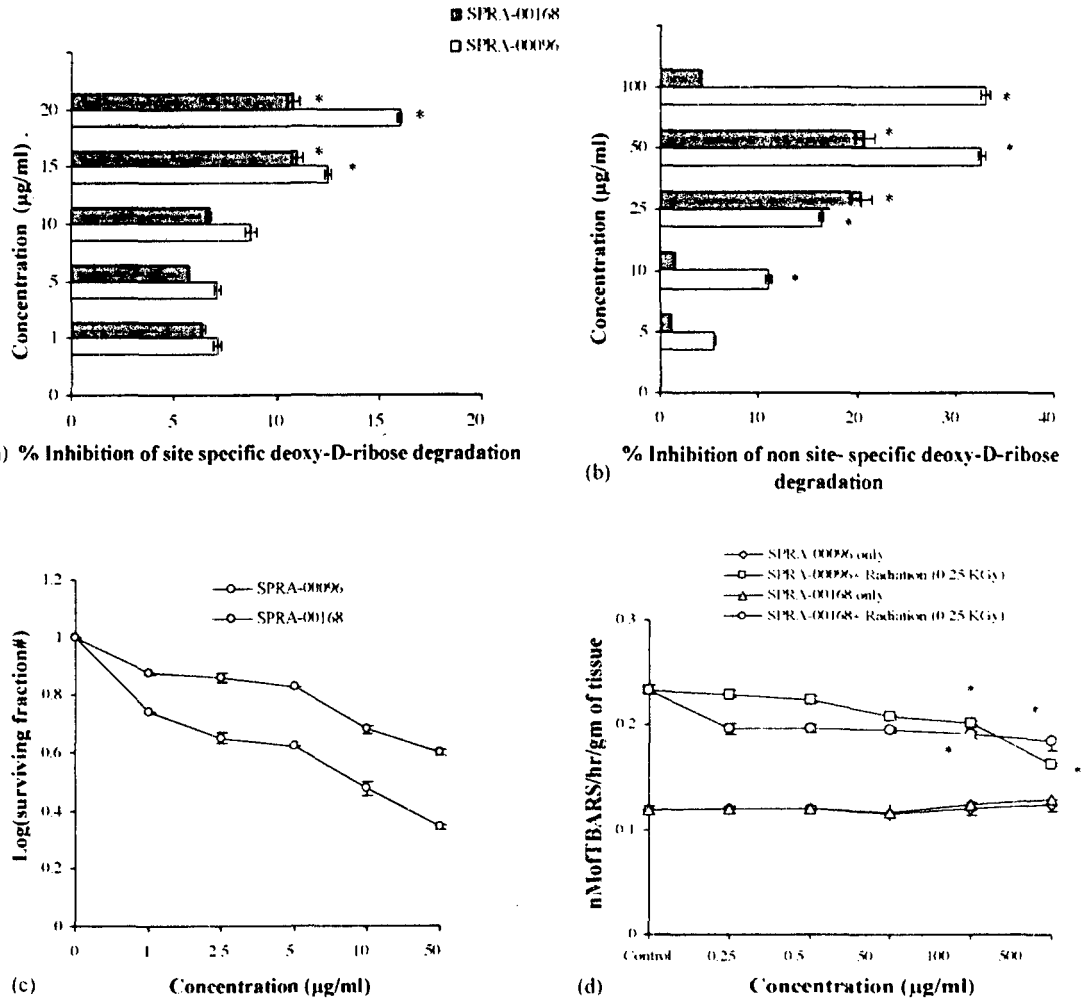


Fig. 5. Evaluation of antioxidant, anti-cancerous and radioprotection property of SPRA-00096 and SPRA-00168: (a) and (b) site-specific and non-site specific •OH ion scavenging of SPRA-00096 and SPRA-00168 activity evaluated in terms of % inhibition (mean ± S.D.) of deoxy-D-ribose degradation as compared with controls (containing no drug) using Student's *t*-test, (c) comparative evaluation of log (cell surviving fraction) of cell-line U-87 vs. different treatments of SPRA-00096 and SPRA-00168, #log(10) = 1 is considered equivalent as 100% survival in control samples, evaluation of lipid peroxidation activity as nM TBARS/h/g of tissue homogenate in liver homogenate against radiation (0.25 kGy) with varied treatments (0.25–500 µg/ml) of SPRA-00096 and SPRA-00168. Standard deviations are indicated as bars. The statistical significance of results was confirmed at 5% level of significance using One Way ANOVA model involving drug + radiation group vs. drug only (containing untreated and radiation control), $^{*}p$ (<0.05) is considered to level of significance.

results directly to deoxyribose prior to hydroxyl generation (Aruouma et al., 1987). On the other hand, the hydroxyl ion scavenging potential was found to increase concomitantly with increase in concentration (0–20 µg/ml) of SPRA-00096 and SPRA-00168 (Fig. 5). The maximum percent inhibition of SPRA-00096 and SPRA-00168 was 15.99% (20 µg/ml) and 10.80% (20 µg/ml), respectively. SPRA-00096 exhibited 5% higher site-specific hydroxyl ion scavenging activity as compared to SPRA-00168, but lesser as compared to non-site specific activity.

3.5. *In vitro cytotoxic analysis*

Cell-line U-87 (neuronal glioma cell line) was used as a model system to evaluate the comparative cytotoxicity of SPRA-00096 and SPRA-00168. SPRA-00096 showed significant decrease in surviving (%) in a dose

range of 1–50 μg/ml and potent cytotoxicity (two times higher cytotoxicity at 50 μg/ml) was exhibited as compared to SPRA-00168.

3.6. Effect of selected fractions during radiation-induced lipid peroxidation studies

The protective effect of aryl tetralin-enriched fractions against supra-lethal radiation-induced membrane damage in Strain 'A' mice liver homogenate was also examined. The anti-lipid peroxidation ability against supra-lethal radiation exposure to mice liver homogenate of SPRA-00096 was observed to be nearly 10% higher as compared to SPRA-00168. No significant change in lipid peroxidation activity at the concentration range tested in case of drug only group was observed as compared to the untreated control. Maximum inhibition (at 500 μg/ml) was found to be 30.21% and 20.60% for SPRA-00096 and SPRA-00168, respectively (Fig. 5). In order to evaluate the range of concentration for which fraction exhibited potent anti-lipid peroxidation activity, a ratio of "anti-lipid peroxidation activity exhibited by the fraction at lowest concentration" to the "anti-lipid peroxidation activity exhibited by the fraction at the highest concentration tested" was calculated. This ratio was found to have an inverse relation to that of range of action, i.e., lower the ratio, higher will be the range of action of a particular fraction. On comparing their ratios, SPRA-00168 (1.06) was found to have a comparable range of action as compared to SPRA-00096 (1.40).

4. Discussion

In this paper, we report for the first time to the best of our knowledge, the production of aryl tetralin lignans by an endophytic fungus. *T. hirusta*, isolated from the rhizomes of high altitude *P. hexandrum*, has been shown to produce bioactive aryl tetralin lignans. The discovery that fungi can bio-synthesize aryl tetralin lignans, including podophyllotoxin, increasingly lends support to the possibility of horizontal gene transfer between *Podophyllum* spp. and its corresponding endophytic organism (Young et al., 1992). The consistent production of aryl tetralin lignan like podophyllotoxin, podophyllotoxin glycoside and demethoxypodophyllotoxin by *T. hirsuta* further supports the theory of Young and co-workers that during the course of evolution, the symbiotic endophytes developed machinery to biosynthesize and tolerate high levels of secondary metabolites in order to better compete and survive in association with the medicinal plant. Previous workers have reported the production of the antileukemic and antitumor drug taxol from the endophytes of *Taxus* spp. like *Taxomyces andreanae* and *Pestalotiopsis microspora* (Stierle et al., 1993; Strobel et al., 1996a,b). Others workers have reported the production of anti-fungal compounds by endophytes (Liu et al., 2001).

In this study, the biological importance of the fungal originated aryl tetralin lignans possessing anticancerous, antioxidant and radioprotective properties was established. The fractions collected at different time intervals exhibited significant ($p < 0.05$) metal chelation and free radical scavenging activities as the inherent ability of aryl tetralin lignans present in the extracts to chelate transition metal ions by filling its aqua coordination sites indicating that both samples are able to boost the natural defence system which includes secondary antioxidants like transferrin and ceruloplasmin (Gutteridge, 1985; Kumar and Goel, 2000). Our earlier report on the ability of plant lignans about their ability to modulate the hemopoietic system under the state of radiation stress via free-radical mediated mechanisms supports the paramount importance of these aryl tetralin lignans (Sagar et al., 2005). Other workers have also reported that other phyto-constituents like silymarin, catechin and luteolin chelate metal ions, and thereby rendering radioprotection (Duthie et al., 2000; Gebhardt, 2002; Korina and Afana'ev, 1997; Morel et al., 1993; Ramadan et al., 2002). Similar results have been observed with *P. hexandrum* and *Rhodiola imbricata* in terms of chelation of metal ions and scavenging of free radicals (Chawla et al., 2005b; Arora et al., 2005b,c). Further, the results indicated that in case of SPRA-00096, PDT and its glycoside contributed to the hydroxyl ion scavenging activity while the substantial increase in DMP and complete absence of PDT glycoside in SPRA-00168 reduces its hydroxyl ion scavenging activity. The loss of PDT glycoside in SPRA-00168 supports the fact that due to low $X \log P$ value (octanol–water partition coefficient) of PDT glycoside (=0.508) makes its more soluble in aqueous system, thereby contributing to hydroxyl ion scavenging activity in aqueous media. The potential of using endophytes

as an effective alternative or novel source for therapeutic compounds has been recognized. Several workers have reported the usage of endophyte fungi from yew trees for production of paclitaxel, an active component of Taxol (Strobel and Long, 1998; Strobel et al., 1996a,b). *T. hirsuta* has been used for the decolorization of a wide variety of dyes, including textile dyes (Rodriguez et al., 2005; Moldes et al., 2003; Campos et al., 2001; Abadulla et al., 2000) as a ligniperdous fungi for production of xylanase (Kubackova et al., 1976), and for microbial metabolism of organo-sulfur compounds, which are of interest in the petrochemical industry for in-field viscosity reduction and desulfurization (Van Hamme et al., 2003). The laccases from *T. hirsuta* have been reported to possess superior characteristics such as high stability, high activity and low carbohydrate content, making it an attractive object for applications in different areas of biotechnology (Shleev et al., 2004).

The tumour–inhibitory properties of PDT and its congeners is well known (Jackson and Dewick, 1985; Broomhead and Dewick, 1990) and we have also established the anti-cancer potential of aryl tetralin lignans-enriched fractions, thereby reducing the dependance on plant species. SPRA-00168 exhibited higher non-toxic range, which could be attributed to the substantially low content of podophyllotoxin in this fraction (Fig. 5). These findings suggested that PDT, PDTG and DMP are required in an appropriate ratio to exhibit significant radioprotection while the anti-carcinogenic activity is exhibited when PDT is maximally present. These results are in corroboration with our previous findings on the radio-protective efficacy of fractionated extracts of *P. hexandrum* (Chawla et al., 2005a; Arora et al., 2005a). The genus *Trametes* has also been reported to produce several compounds of pharmaceutical importance. Several antitumor compounds, mainly polysaccharides, e.g., Krestin (PSK in Japan; PSP in China) have been developed and commercialized using the submerged mycelial biomass of *T. versicolor* (Hiroshi and Takeda, 1993). Krestin is approved for use against a number of cancers, including cancers of the stomach, oesophagus, rectum, lung, mammary gland cancer, reductions of side-effects of chemotherapy and comprised 25% of the anticancer drug market in Japan and the total annual sales exceeded US$ 350 million in the year 1996 (Mizuno, 1996). *T. versicolor* has also been reported to transform very high concentrations of recalcitrant pollutants such as chlorophenols—are of the most dangerous classes of environmental pollutants that are produced in very large quantities (thousands of tonnes) every year by the pulp and paper, and agrochemical and pharmaceutical industries world-over.

Further, the anti-lipid peroxidation ability of aryl tetralin lignans enriched fungal fractions against supralethal radiation induced stress was also investigated. The higher anti-lipid peroxidation activity of SPRA-00096 could be attributed to its higher hydroxyl-ion scavenging potential as compared to SPRA-00168, as well as due to its constituents like PDT and its glycoside. On the other hand, the increased levels of demethylpodophyllotoxin in SPRA-00168 could be a contributing factor to enhanced range of action, as its activity ratio is slightly lower as compared to SPRA-00096. The comparative activities of two fractions is more or less to depends upon the permeation and solubility properties of numerous constituents which could be explainable by their Lipinski values (Lipnski et al., 1997). Both PDT and 4′-demthyl PDT has $X\log P$ (octanol–water partition coefficient) 2.01 and 1.96 values, respectively. In addition to this, the number of hydrogen bond acceptor sites are less than 10 as well as their molecular weight is less than 500 Da, which puts both of these chemical compounds into the category of compounds with good permeation properties. This further supports their ability to protect lipid membranes against radiation-induced peroxidative stress. On the other hand, PDT glycoside increases the range of action being more soluble in aqueous system as compared to the other two, thereby reduces the free radical flux within aqueous system. This finding could be further corroborated to the reduction in hydroxyl ion scavenging potential of fraction SPRA-00168 due to the loss of PDT glycoside. These results could be further corroborated with the earlier findings by our group in a comparative evaluation of the fractionated extracts of *P. hexandrum* containing aryl tetralin lignans (Chawla et al., 2005a). These results of our investigation confirm that the aryl tetralin lignans synthesized by the endophyte *T. hirsuta* possesses antioxidant, anticancer and radioprotective properties.

Studies are underway to standardize media and growth conditions in bioreactors/fermentor for upscaling, and preliminary results have been very encour-

aging (unpublished data). The fermentor was optimized under continuous shaking (400 rpm) and acidic pH of the culture. The final pH and dissolved oxygen at the time of termination were recorded as 4.0 and 58%, respectively. Further refined studies in large-scale fermentor are likely to result in an efficient process for production of secondary metabolites of *Podophyllum* in large quantities that could be utilized for industrial production of this important group of compounds. The results indicate that *T. hirsuta* is an excellent candidate for consideration in fermentation technology. The present study has focused only on the production of aryl tetralin lignans by *T. hirsuta*, however, the implications of the present study in environmental biotechnology particularly for the bioremediation of contaminated soils and the disposal of chemical wastes (Sedarati et al., 2003; Steieret and Crawford, 1985) remains a virgin area that needs to be explored. The next step will be expand and apply the work at various levels to other areas of biotechnology. We expect that biotechnological application of the endophyte isolated will be in multiple areas.

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